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THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF IRON-REDUCING *ALTEROMONAS PUTREFACIENS* STRAINS ISOLATED FROM OIL FIELD FLUIDS AND THEIR ROLE IN CORROSION

BY

KATHLEEN MARGARET SEMPLE CHOW

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1987

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ABSTRACT

Gram-negative, facultative, aerobic bacteria capable of using ferric iron, thiosulfate or sulfite as electron acceptors under anaerobic conditions were readily isolated from oil field fluids in north central Alberta. These bacteria are widely distributed, representing a major component (up to 39%) of the recoverable aerobic bacterial population in some oil fields and have been implicated in the corrosion of metal in the oil field environment. Morphological and biochemical data support the classification of these isolates as strains of *Alteromonas putrefaciens* (mol% G+C 42-56). The 80 isolates characterized expressed a marked degree of halotolerance, as some grew in the presence of 7.5% sodium chloride and some grew well at 4°C. Isolates representing all of the four DNA homology groups of *A. putrefaciens* were isolated from oil field fluids. All isolates reduced soluble ferric iron at approximately the same rate (7.5 to 42.5 μmol Fe (II)/h/mg protein). Those isolates tested also were able to release ferrous ions from various forms of insoluble ferric iron oxides, with amorphous oxides being reduced preferentially to crystalline oxides. Data are presented on hydrogen sulfide production during anaerobic growth and sulfite reduction by four isolates. Analysis of the sulfur isotope composition ($\delta^{34}S$) of the evolved sulfide shows normal and inverse isotope fractionation patterns characteristics of a dissimilatory sulfite reduction pathway. The ability to reduce ferric oxide and produce sulfide, and their ubiquitous presence in the oil field environment, indicates that these bacteria could contribute to corrosion of metals used in the oil industry.

The role of iron reducing bacteria in the corrosion process and their effects on corrosion by sulfate-reducing bacteria was investigated by following the attachment to and corrosion of mild steel coupons. Pure and mixed cultures of *A. putrefaciens* isolate Ps 200 and a sulfate-reducing isolate *Desulfovibrio vulgaris* AL1 were grown in different media under continuous culture conditions. Generally, even if good growth and sulfide

generation occurred in the culture, high corrosion rates and extensive corrosion were only observed in the cases where the scanning electron microscopy studies showed attachment of the bacteria and the formation of a black, iron sulfide-rich biofouling layer on the surface of the coupons. Localized corrosion was seen under loosely adhering areas of the biofilm. Growth in mixed cultures of both isolate Ps 200 and the SRB greatly affected the amount of biofouling which developed on coupons in the different media used and hence the degree of corrosion observed, as compared to that observed in monocultures. The presence of yeast extract in a medium resulted in a decrease in the amount of biofilm formation whereas an increase in biofouling was seen in a medium without yeast extract. The highest corrosion rate (22.7 $\pm$1.3 mg dm^{-2} day^{-1} or 4.2 mils per year) and noticable pitting of the coupons was observed with mixed cultures in the absence of yeast extract.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
Bd	Bouyant density
IPL	Interprovincial Pipe Line
mdd	mg dm^{-2} day^{-1}
mpy	mils per year
MPN	most probable number
NACE	National Association of Corrosion Engineers
PCA	plate count agar
ppt	parts per thousand
SEM	scanning electron microscope
SRB	sulfate-reducing bacteria
Tm	thermal melting point
TMA	trimethylamine
TMAO	trimethylamine oxide
YE	yeast extract

1 INTRODUCTION

Bacteria are closely associated with many aspects of petroleum production (Davis 1967; Chater and Somerville 1978; Moses and Springham 1982; Atlas 1984) and are important in the corrosion of metal used in oil field systems. Microorganisms, especially sulfate-reducing bacteria (SRB) have a widespread impact in the petroleum industry by corroding pipelines, oil storage tanks, pumping and water injection systems, steel casings and refinery equipment. The costs of corrosion are significant, with an estimated 50% of pipeline failures due to biologically enhanced corrosion and billions of dollars spent each year replacing and maintaining pipelines (Miller 1981; Kendrick 1984). The true extent of microbial corrosion problems is only slowly being recognized, and was the subject of a National Association of Corrosion Engineers conference in 1985 (Dexter 1986).

Sulfate-reducing bacteria are the preeminent bacteria responsible for corrosion problems and most research concerns the mechanisms of anaerobic corrosion of iron and steel by these organisms. These bacteria, however, represent only a part of the flora associated with oil production as, in addition to the classical corrosion-causing SRB, large numbers of facultative and aerobic organisms are also isolated from oil, produced water and corroding pipe (Westlake and Cook 1978). Among the deficiencies in corrosion research identified by Pope et al. (1984) was the need to understand the role of these other organisms in microbiologically influenced corrosion, their mechanisms of corrosion and to study their interaction with the SRB in mixed communities.

Corrosion has been defined by Fontana and Greene (1978) as the destruction of a material through reaction with its environment. Corrosion, as a strictly electrochemical process, involves the maintenance of an electrochemical cell, on metal immersed in an electrolyte, in which the anodic reaction involves the oxidation and dissolution of the metal and the cathodic reaction requires a reduction reaction. Possible reductants are oxygen, under aerobic conditions, hydrogen ions under acidic or anaerobic conditions or

possibly reducible metal ions in solution (i.e. $Fe^{3+} + e^- \rightarrow Fe^{2+}$).

ANODE	$Fe^\circ \rightarrow Fe^{2+} + 2e^-$	
CATHODE	$O_2 + 2H_2O + 4e^- \rightarrow 4\ OH^-$	aerobic/neutral
	$2H^+ + 2e^- \rightarrow 2H \cdot \rightarrow H_2$	acids/anaerobic

The electrochemical reactions must proceed simultaneously and be sustained for an extended period at the anodic (corroding) and cathodic zones on the metal surface for corrosion damage to occur.

Fontana and Greene (1978), and NACE (1984) are two excellent reference books which discuss the background and theories of corrosion. Some of the basic concepts important to the understanding of microbiologically influenced corrosion will be discussed briefly. As a metal oxidizes, a thin layer of oxide or corrosion product may build up on the surface. The small amount of generalized corrosion which occurs can prevent further oxidation, through a process referred to as passivation. For example, passivation by the formation of a thin oxide layer is the basis for the corrosion resistance of stainless steel. Pitting corrosion can take place if this protective layer is disrupted (depassivation) in localized areas. The exposed metal surface becomes the anode, and corrodes, while the remaining surface functions as the cathode. Oxygen is quickly used up inside a corroding 'pit', while the outside cathode is still aerated. The hydrogen and chloride ion concentrations increase inside the pit and the process becomes autocatalytic. Similar mechanisms of corrosion are active in crevices or when deposits are formed on the metal surface, where corrosion occurs under the less oxygenated areas (anodes). When areas on the metal surface are under different corrosion conditions, oxygen concentration cells or other differential chemical concentration cells form, which create very corrosive conditions and accelerate the corrosion process. Galvanic corrosion can take place when two unlike metals are in contact, where one is more easily oxidized than

the other and becomes the anode (corrodes) while the other acts as the cathode.

Microbial corrosion does not involve any new mechanisms of corrosion, but refers to the involvement of the microbes in initiating and accelerating the electrochemical process. In fact, bacterial corrosion is most obvious under conditions that were not originally expected to be corrosive, such as in fresh water at neutral pH, or under anaerobic conditions with underground pipes. For example, according to the classical cathodic depolarization theory (Von Wolzogen Kuhr 1961), SRB may be directly responsible for enhancing corrosion anaerobically by removing the cathodic hydrogen which builds up on metal surfaces. Many types of organisms have been isolated from suspected microbial corrosion sites (Stoeker 1984) and the various corrosion mechanisms, including those for iron-oxidizing and sulfur-oxidizing bacteria, have been reviewed by Pope et al. (1984). A number of compiled lists of the general mechanisms of biologically-induced corrosion have been prepared (Miller 1981; Pope et al. 1984; Tiller 1986; Dexter 1986; Ford et al. 1986) and they include the following:

(1) Direct influence on the cathodic or anodic reaction. i.e. interference with the cathode reaction under oxygen-free conditions by the organisms (SRB) and metabolic sulfides.

(2) Creation of differential aeration cells or chemical concentration cells by absorption of nutrients (i.e. O_2) by growing microbial deposits on the metal surface or formation of galvanic couples and ion concentration cells by chelation of metal ions.

(3) Production of corrosive metabolic products such as organic or inorganic acids and sulfide.

(4) Prevention of the formation or destruction of passive or protective films.

(5) Destruction or inactivation of corrosion inhibitors, biocides, coatings or lubricants. Biofilms may also prevent corrosion inhibitors from reaching active corrosion sites on the metal.

Among the facultative isolates frequently found in fluids produced from oil fields using water flooding techniques are gram-negative, facultative pseudomonads capable of anaerobic growth using a few low molecular weight carbon sources (e.g. lactate) and ferric iron or partially reduced forms of sulfur, but not sulfate, as an electron acceptor (C.O. Obuekwe. 1980. Ph.D. thesis, University of Alberta, Edmonton, Alberta). When growing aerobically, these strains are able to use a wide range of carbon sources to support growth. Such isolates are aerobes, oxidase-positive, nonspore-forming, motile rods with a polar flagellum and were classified as being members of the genus *Pseudomonas*. The implications of the activities of this group of organisms in the corrosion of metals used in the petroleum industry has recently been reviewed (Westlake et al. 1986). Obuekwe (Ph.D. thesis) extensively studied the characteristics of the iron reduction system (Obuekwe et al. 1981a; Obuekwe and Westlake 1982a, 1982b) and the generation of sulfide (Obuekwe et al. 1983) by these bacteria. In a series of corrosion studies, Obuekwe et al. (1981b, 1981c, 1981d, 1983, 1987) concluded that this type of bacteria could contribute to corrosion by:

(1) Anodic depolarization by reducing and disrupting the protective ferric iron films on the metal.

(2) Increasing the ferrous iron concentration by reduction of ferric iron compounds thus increasing the production of iron sulfide.

(3) Attachment to and colonization of the metal surface by exopolysaccharide production thus creating differential aeration and concentration cells.

(4) Creating reduced conditions for the growth of SRB under the attached biofilm.

(5) Enhancing sulfide production using intermediates of sulfate reduction, possibly in synergy with the SRB in mixed consortia.

There is little information available about the distribution and characterization of

iron-reducing bacteria. However, before these organisms can be implicated in corrosion of pipelines, production and storage equipment, their occurrence in the oil field environment has to be investigated.

In addition to their potential role as corrosion-causing bacteria, it is important to evaluate these organisms in terms of their role in biogeochemical iron and sulfur cycles. Obuekwe's isolates have been reported to have an exceptional ability to reduce ferric iron and the mechanism of iron reduction by one isolate, Ps 200, has been investigated by Arnold et al. (1986a, 1986b). Obuekwe et al. (1983) investigated sulfite and thiosulfate reduction by strains of these bacteria, and proposed a cascade theory of sulfide generation, based on a dissimilatory sulfite reduction pathway in these organisms. Sulfite and thiosulfite reduction have previously been reported for SRB (Postgate 1984), clostridia (Laishley et al. 1984) and fermentative organisms such as *Salmonella* spp. and *Proteus* spp. (Oltmann et al. 1975). The nature of the sulfite reduction by facultative aerobic strains needs to be investigated. Do they use a pathway similar to that used by SRB and do they have the ability to grow anaerobically in synergism with other bacteria? Characterization and identification of these bacteria would help to determine if organisms with the combined ability to reduce ferric iron and sulfite are metabolically a unique type of bacteria and to compare the distribution, characteristics and economic activities of the oil field isolates to known strains of bacteria.

This study, therefore, was undertaken to investigate the role of iron-reducing bacteria in the corrosion process and to determine their influence on the corrosion by SRB in mixed culture. The objectives were:

(1) To study the distribution and occurence of iron-reducing bacteria in produced water and oil samples from producing oil fields in Alberta.

(2) To isolate, characterize and identify strains similar to Obuekwe's organisms to confirm a correlation between iron reduction and sulfide generation by these bacteria.

(3) To compare the rates of ferric iron reduction of Obuekwe's isolate Ps 200 to

the new strains isolated and to test their ability to reduce various forms of insoluble ferric iron oxides.

(4) To test isolates for the ability to reduce sulfite and thiosulfite and to evaluate selected isolates for their ability to fractionate sulfur isotopes during anaerobic growth and sulfite reduction.

(5) To assess the ability of isolate Ps 200 to attach to and corrode mild steel coupons, in pure and mixed culture with a strain of SRB, in continuous culture under iron-reducing and sulfide-generating conditions.

II LITERATURE SURVEY

A. BIOLOGICALLY-INFLUENCED CORROSION

Microbial corrosion in Alberta oil fields

Our interest in microbial corrosion focuses on the petroleum industry in Western Canada. Extensive pipeline systems throughout the province link producing oil fields with storage facilities, such as Interprovincial Pipe Line (IPL) Terminal, Edmonton, Alberta, Canada which then transport oil to refineries in eastern Canada and the USA. Pipeline failures due to corrosion result in appreciable economic loss to this industry.

Frequent corrosion problems have been recorded in pipelines and production equipment in oil fields using secondary recovery techniques such as water injection to maintain oil production. Corrosion failures in these areas are associated with a large bacterial load which probably contributes to the corrosion (Westlake and Cook 1978). Extensive monitoring of fluids from producing wells, pipelines and storage tanks in oil fields such as Pembina in northern Alberta over the past 10 years has shown a constant occurrence of a variety of bacteria (C.O. Obuekwe, Ph.D. thesis). In addition to the ever-present anaerobic SRB, there was a thriving population of aerobic and facultative anaerobic bacteria. Microbiological surveys of the IPL system from Edmonton to Wisconsin found iron-reducing, sulfide-generating bacteria throughout the system (Westlake and Cook. 1976. University of Alberta, Edmonton, Unpublished data). Iron-reducers were thought to enhance corrosion by solubilizing iron atoms while sulfite-reducing bacteria were implicated in corrosion by enhancing sulfide generation in the system. High corrosion rates in the field are likely due to the activity of the microbial community acting together to accelerate corrosion (Westlake and Cook 1978). Research was undertaken in an attempt to understand the corrosive activities of other components of the mixed microbial community in the overall corrosion process (C.O. Obuekwe, Ph.D. thesis).

Corrosion by sulfate-reducing bacteria

The problems associated with SRB in the oil industry have been described by many authors (NACE 1982; King and Stott 1983; Hamilton 1983b; Iverson and Olson 1984; Kendrick 1984; Postgate 1984; Maxwell and Hamilton 1986; Sanders and Hamilton 1986; Dewar 1986; King et al. 1986; Cord-Ruswisch et al. 1987). Microorganisms, especially SRB, have a widespread impact on the petroleum industry by corroding pipelines, oil storage tanks, pumping and water injection systems, steel casings and refinery epuipment. They are also implicated in souring the oil formations and petroleum products, thus increasing the cost of hydrocarbon recovery. The hydrogen sulfide produced is also highly toxic and thus a potential health problem for oil field workers. Bacterial products can also cause plugging of the oil-containing formation.

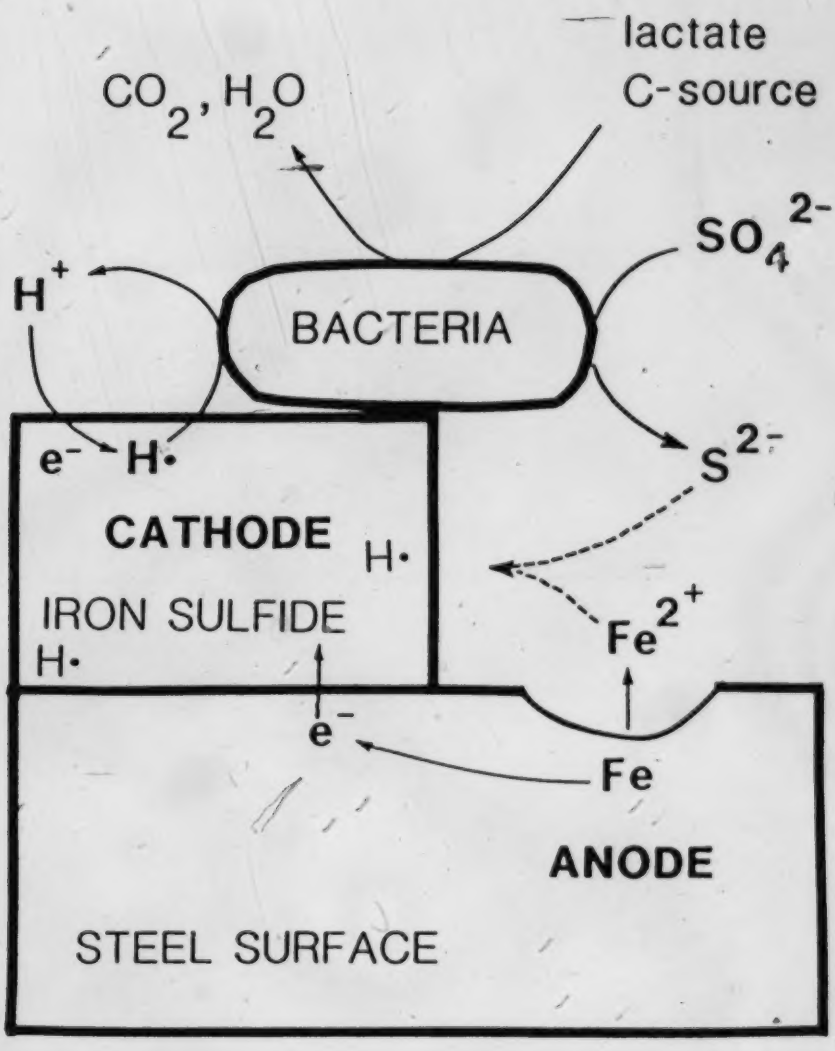
The role of SRB in corrosion has been extensively studied and these bacteria are monitored in the oil field fluids as an index of the presence of corrosion-causing organisms. Extensive literature exists on the possible mechanisms of corrosion by SRB: these mechanisms have been the subject of many excellent reviews (Miller and King 1975; Iverson 1981; Miller 1981; Cragnolino and Tuovinen 1983; Tiller 1982, 1983, 1986). Their activity is related to the maintenance of a corrosion cell by the production of corrosive ferrous sulfides (Booth et al. 1967; King and Miller 1971; Mara and Williams 1972; King et al. 1973a; King and Wakerly 1973) and the uptake of cathodic hydrogen by hydrogenase activity. The proof of the classical cathodic depolarization theory advanced by von Wolzogen Kuhr (1961) has been reviewed by Miller and Tiller (1971) and Tiller (1982, 1986). Electrochemical studies have shown that corrosion current is proportional to hydrogenase activity in low iron medium (Booth and Tiller 1960, 1968; Booth and Wormwell 1962; Iverson 1966). However, in high iron medium, hydrogenase-negative strains had the same corrosive activity as hydrogenase-positive strains if unprotective films of iron sulfide formed (Booth et al. 1967, 1968). Recent studies have proven that hydrogenase-positive *Desulfovibrio* can use cathodic H_2 from

metal as energy source for growth with acetate (Hardy 1983; Pankhania et al. 1986a, 1986b; Tomei and Mitchell 1986). Costello (1974) proposed that the reduction of H_2S was the more important cathode reaction which caused cathodic depolarization rather than the reduction of H^+ to H_2. However, all of the possible cathode reactions which are suggested for sour gas environments (Ogundele and White 1986), involving the reduction of HCO_3^-, H_2S, HS^- or H^+, produce H_2, and thus could be influenced by SRB. Most workers regard the mechanism of cathodic depolarization resulting from the removal of hydrogen by hydrogenase as contributory to the more important role of iron sulfides in corrosion (Miller and Tiller 1971; Smith and Miller 1975; Miller and King 1975).

The role of iron sulfides in corrosion has been reviewed by Tiller (1986, 1982) and Hamilton (1985). Iron sulfides are also cathodic to the metal by absorbing hydrogen and can create a galvanic corrosion cell between the iron and FeS. The model of King and Miller (1971) (Figure 1) incorporates both the role of hydrogenase and the Fe°/FeS galvanic cell in corrosion. Chemically prepared iron sulfides are corrosive to metal by absorbing hydrogen from the surface (Booth et al. 1968; King and Wakerly 1973), but are not a permanent cathode and must be continually replaced (King and Miller 1971; King et al. 1973a). If SRB are present, the corrosion cell is maintained by regenerating the cathode through the use of the hydrogen in the FeS matrix or by generating new FeS by producing sulfide.

The physical and chemical form of the iron sulfide film greatly affects the corrosion rate, determining whether it is protective or unprotective to the metal (Mara and Williams 1972; King and Wakerly 1973). The properties of the various iron sulfide forms (i.e. mackiniawite [$Fe_{1-x}S$]; greigite [Fe_3S_4], etc.) related to corrosion, are reviewed by Smith and Miller (1975). When unprotective FeS films are formed, such as in high iron medium, corrosion rates are very high (Booth et al. 1967; Mara and Williams 1972; King, Miller and Wakerly 1973b). In low iron media, initially protective films are

Figure 1. Proposed mechanism of anaerobic microbial corrosion involving iron sulfide. Based on Miller and King (1971) and Smith and Miller (1975).


lactate
C-source
CO_2, H_2O
SO_4^{2-}
H^+
BACTERIA
e^- H·
CATHODE
H·
IRON SULFIDE
H·
S^{2-}
Fe^{2+}
e^-
Fe
ANODE
STEEL SURFACE

formed, which may then break down and become unprotective. The film differentiates into anodic and cathodic areas, with enhanced corrosion taking place under loosely adherent films (Mara and Williams 1972; Hardy and Brown 1984). Differentiation and breakdown of the film in low iron medium depends on bacterial activity, perhaps by increasing the sulfur content, which in turn changes the crystal structure of the FeS (King and Miller 1971; Mara and Willams 1972).

Alternating aerobic and anaerobic conditions are extremely corrosive and tend to reflect the corrosion conditions found in the field (Hamilton 1985; Tiller 1986). Hardy and Brown (1984) reported that a substantial increase in corrosion takes place when biogenic sulfide films are exposed to air, which may account for the high corrosion in the field. Newman et al. (1986) suggested that thiosulfite formation, in sulfate medium, might explain the pitting seen under aerated biogenic sulfide films. Elemental sulfur, produced by the oxidation of sulfide, is also very corrosive (Farrer and Wormwell 1953; Schaschl 1980). The formation of and corrosion by various corrosive sulfur anion intermediates is reviewed by Cragnolino and Tuovinen (1983). Oxygen concentration cells would be active in aerated conditions as well, where the metal shielded by the biofilm becomes anodic relative to the exposed, aerated cathode areas.

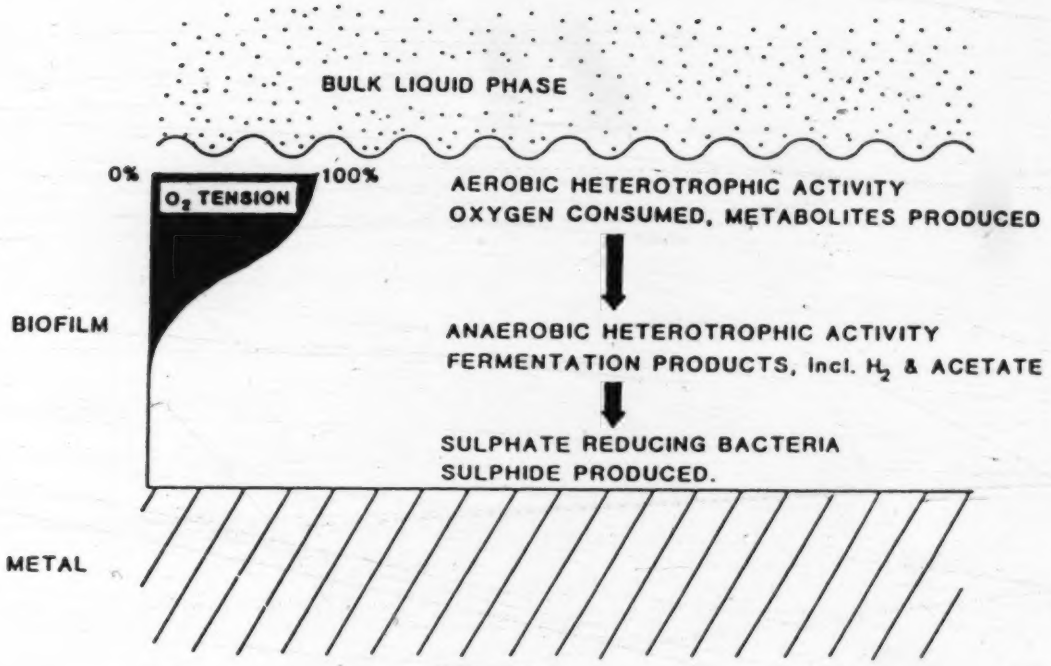
An additional mechanism for corrosion by SRB involving a highly corrosive, volatile phosphorus compound has been suggested by Iverson (Iverson 1981; Iverson and Olson 1983; Iverson et al. 1986). Black iron phosphides were identified in corrosion products isolated from laboratory experiments using pure cultures of SRB in sulfate-free medium. The importance of this mechanism in the field and under sulfate-reducing conditions needs to be investigated further. The apparent controversy in the literature, concerning which mechanisms are more significant, actually emphasizes the fact that corrosion in the field is a very complex system with the possiblility of a number of mechanisms functioning in each situation (Starkey 1986).

Importance of mixed cultures to corrosion

The importance of mixed communities of organisms in the corrosion of metals has been emphasized in a number of reviews by Hamilton (1983a, 1983b, 1985). However, only a few systematic studies have been carried out on the effects of mixed populations on corrosion by SRB (Gaylarde and Johnston 1982,1986).

The characteristics of SRB have been described by Postgate (1984). They are ubiquitous, being found in sediments, mudsand sewage, as well as in oil production, transport and storage facilities, where hydrocarbons are the primary nutrient. These organisms cannot use oil directly, so they depend on aerobic, oil-degrading bacteria to generate the substrates for growth (Jobson et al. 1979). The classical SRB, *Desulfovibrio* and *Desulfotomaculum*, use a limited number of low molecular weight carbon sources for growth, such as lactate, and produce acetate. The recent studies of Pfennig and Widdel (Pfennig et al. 1981) have described a large number of 'new' morphological and physiological types of SRB with a broad nutritional range. They grow on a wide range of organic compounds, including benzoate and fatty acids, from acetate to long chain acids. The implications of these new physiological groups of SRB to corrosion study and monitoring has been reviewed by Hamilton (1983a, 1983b, 1985). Sulfate-reducing bacteria are present in oxygenated environments, such as the ocean, and vary in their tolerance to oxygen (Cypionka et al. 1985) but grow only under anoxic conditions. They are therefore dependent on other bacteria to provide a reduced environment and suitable nutrients for their growth and tend to occur as components of a "consortia" of organisms, often in the form of a biofilm (Hamilton 1983a, 1983b, 1985; Costerton and Geesey 1986; Sanders and Maxwell 1983). The interactions within the biofilm are important to study if one is to control corrosion and other problems related to the presence of SRB. A model for development of biological corrosion communities involving SRB on the surface of metal (Figure 2) was proposed by Hamilton (1985). He concluded that corrosion processes have their origins in the growth and activities of

Figure 2. Proposed relationships between microorganisms growing in a biofilm on a metal surface (from Hamilton 1985).


BULK LIQUID PHASE
0%
100%
O_2 TENSION
AEROBIC HETEROTROPHIC ACTIVITY
OXYGEN CONSUMED, METABOLITES PRODUCED
BIOFILM
ANAEROBIC HETEROTROPHIC ACTIVITY
FERMENTATION PRODUCTS, Incl. H_2 & ACETATE
SULPHATE REDUCING BACTERIA
SULPHIDE PRODUCED.
METAL

microbial communities and should be studied as such.

Importance of biofilm to corrosion

The importance of biofilm to corrosion has been emphasized by Hamilton (1985), Obuekwe et al. (1981b) and Costerton (Costerton et al. 1981; Costerton and Lashen 1984; Costerton and Geesey 1986). Tiller (1982) concluded that mechanisms of microbial corrosion are closely associated with the biofouling process.

Case histories of biologically induced corrosion described by Kobrin (1986), Stoeker (1984) and Tatnall (1981a,1981b) indicate that corrosion occurs as localized, pitting sites under characteristic tubercles, deposits or films of bacteria. Corrosion related to SRB has been characterized by localized or pitting corrosion beneath black iron sulfide slime which, when removed, reveals bright metal pits (NACE 1982; Tiller 1982; Hamilton 1985). Testing methods for monitoring for biological corrosion in oil pipelines emphasize the importance of testing for attached microbes using surface evaluation techniques (Chen and Chen 1984, King et al. 1986, Costerton and Geesey 1986). Monitoring the number of planktonic bacteria does not give an indication of the numbers or activity of the attached population (King et al. 1986; Sanders and Maxwell 1983; Maxwell and Hamilton 1986). Effective corrosion control methods used in pipelines include scraping the surface (pigging), especially in stagnant areas, to remove deposits, corrosion products and the attached bacteria (NACE 1975a; King et al. 1986). Testing and use of biocides for corrosion control must be directed against the attached populations of bacteria because of the resistance of a biofilm to biocide penetration (Ruseska et al. 1982; Costerton and Lashen 1984).

Gaylarde and Johnston (1980) studied the importance of attachment to corrosion and showed that organisms and/or high molecular weight corrosive compounds must be in intimate contact with the metal for corrosion to occur. Factors affecting the adhesion of bacteria to surfaces, in relation to corrosion, are reviewed by Duddridge and Pritchard

(1983). In the model of King and Miller (1971) (Figure 1), iron sulfides must be in direct contact with the metal to act as a cathode. The requirement for direct contact of the FeS was noted because chemically prepared FeS showed greatest corrosion on horizontal surfaces. Tiller (1982) suggested that this was the only orientation in which FeS would develop. However, an attached biofilm would hold the FeS in place in any orientation and FeS build-up in a biofilm enhances contact between the anode and FeS cathode (Herbert and Stott 1983). This may, in part, explain the increased corrosion seen with sulfide films of biogenic origin.

A number of research groups, concerned with the formation of microbial films on metal, have studied corrosion by organisms other than SRB using techniques designed to study undisturbed films. Exopolymer-producing slime bacteria have been recognized as being important to corrosion by providing reduced microniches for SRB (NACE 1982). Bacterial deposits themselves can also create corrosion conditions by forming differential aeration cells and concentration cells. Exopolymers may have an indirect role in corrosion by concentrating corrosive metal ions (Little et al. 1986; Nivens et al. 1986; White et al. 1986 Ford et al. 1987) as well as by creating O_2, H_2S and H^+ gradients which can drive corrosion (Costerton and Geesey 1986). The thermophilic organisms studied by Little et al. (1986) and Ford et al. (1987) were found to form films on the metal and weaken the passive films. This may involve the production of acid metabolites. Schiffrin and Sanchez (1985) isolated a corrosion-causing *Pseudomonas* sp. from copper based alloys in marine heat exchangers which they identified as a strain of organism associated with the spoilage of marine whitefish. They investigated the depolarizing effect of trimethlyamine (TMA) production and concluded that slime formation by these bacteria depassivates the metal by limiting oxygen diffusion to the surface.

Corrosion by iron-reducing bacteria

Extensive studies by Obuekwe (Obuekwe et al. 1981b; 1981c; 1981d; 1983; 1987) on the corrosion-causing abilities of facultative iron-reducing bacteria were reviewed by Westlake et al. (1986). These organisms produce exopolysaccharides (Obuekwe et al. 1981b) which mediate their attachment to the surface of mild steel coupons and result in the formation of extensive mats of cells after extended incubation periods.

The polarization characteristics of mild steel coupons exposed to iron-reducing isolate Ps 200 were investigated (Obuekwe et al. 1981c, 1981d). These bacteria affected the corrosion rate by the reduction of protective ferric oxide films on the metal surface, causing anodic depolarization as a result of an increased activity or number of anodic sites. The ability to reduce the insoluble, protective ferric iron forms to soluble ferrous ions is very important in the corrosion capabilities of this group of bacteria.

Convincing evidence for the importance of anodic depolarization and film disruption was demonstrated by following the disruption of protective gamma ferric oxide film, generated on metal coupons during nitrate reduction (Obuekwe et al. 1981d). Corrosion by nitrate-reducing bacteria (Ashton et al. 1973) occurred under nitrate-reducing conditions; by producing corrosive nitrite, a protective gamma-ferric oxide coating was formed. After nitrate reduction had ceased, the iron-reducing activity of the organisms resulted in intense anodic depolarization and a concomitant increase in corrosion rate, due to disruption of the film. The increase in corrosion rate followed an increase in the anodic current. The anodic depolarization of mild steel is due to the activity of the iron-reducers on the metal surface and results from their ability to remove the passivating ferric oxide film. This exposes bare metal, thus making the metal more active and enhancing its corrosion. This mechanism may be most effective in the absence of sulfide generation because sulfide chemically reduces the ferric oxide, thus disrupting the passive layer and causing anodic depolarization (Salverezza and Videla 1980; Videla 1986).

Cyclic exposure to alternating aerobic and anaerobic culture conditions resulted in the highest corrosion rates (C.O. Obuekwe, Ph.D. thesis). Alternate formation of an oxide coating on the metal surface and its subsequent removal by the organisms in culture resulted in a corrosion rate of 105 mg dm^{-2} day^{-1} (mdd) or 0.45 mm $year^{-1}$.

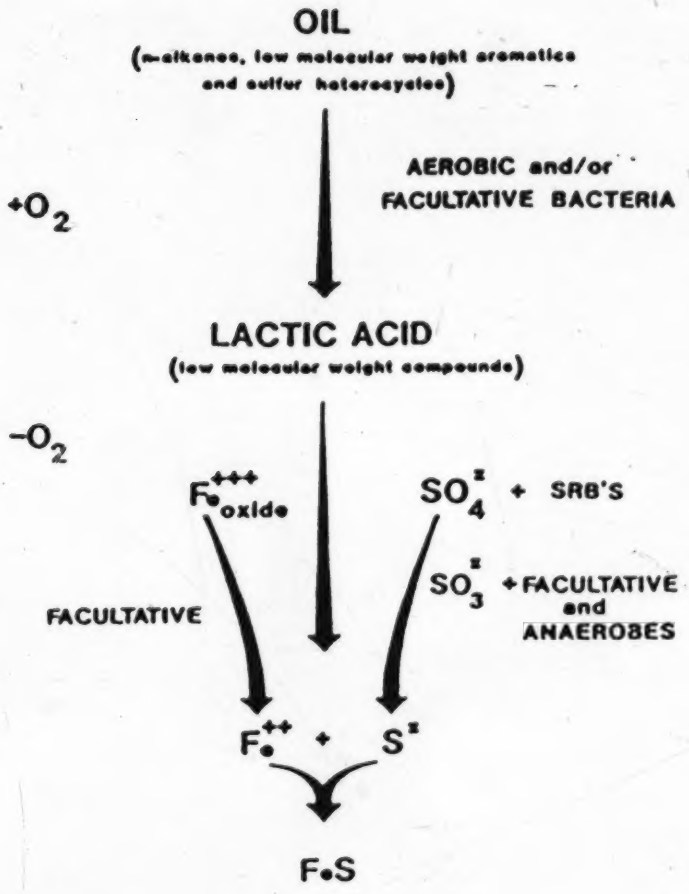
In addition to the ability to reduce ferric iron, these organisms may also be important by virtue of their ability to generate sulfide. Pitting of metal coupons was seen under sulfide-generating conditions when isolate Ps 200 was provided with thiosulfate as a utilizable sulfur source in continuous culture. Removal of the black coating revealed bright pits on the surface (C.O. Obuekwe, Ph.D. thesis). The synergistic interaction of SRB and facultative sulfide-generators may make an active contribution to the generation of hydrogen sulfide in oil field systems (Obuekwe et al. 1983). A model for the interaction of the aerobic, facultative and anaerobic bacteria found in oil field fluids (Figure 3) was proposed by Westlake et al. (1986).

Corrosion study strategy

Several guidelines for corrosion study were suggested by Tatnall (1986) and reiterated by other scientists at the NACE International Conference on Biologically Induced Corrosion (Dexter 1986). In laboratory corrosion studies, it is important to simulate, as closely as possible, the environments in which corrosion takes place.

Corrosion investigations should be carried out with mixed microbial populations in order to assess the contribution of other organisms to the process, such as oil-degrading, biofilm-forming and sulfide-generating bacteria. Ideally, cultures should be isolated from actual microbial corrosion sites. Biological corrosion usually occurs in poor nutrient conditions, i.e. near starvation conditions, which greatly affects the physiology of the organisms and their association with surfaces (Morita 1982; Wardell et al. 1983; Kjellberg 1984; Fletcher 1985). Media rich in organic material and nutrients usually support free floating, not attached organisms, so that a high nutrient synthetic medium which supports

Figure 3. Interaction of aerobic, facultative and anaerobic bacteria present in oil field fluids in the formation of ferrous sulfide and the corrosion of metals. (from Westlake et al. 1986).


OIL
(n-alkenes, low molecular weight aromatics and sulfur heterocycles)
+O2
AEROBIC and/or FACULTATIVE BACTERIA
LACTIC ACID
(low molecular weight compounds)
-O2
Fe+++ oxide
SO4=
+ SRB'S
SO3=
+ FACULTATIVE and ANAEROBES
FACULTATIVE
Fe++
+
S=
FeS

good growth of the organisms does not necessarily produce microbial corrosion (Tatnall 1986). Dr. R.A. King, in a presentation at the NACE Meeting 1985 (Proceedings: Dexter 1986) suggested that yeast extract, which is usually included in media formulations, should be excluded from corrosion testing media because it "inhibits" corrosion. Most corrosion studies have been carried out with anaerobic conditions imposed, but corrosion often occurs under aerobic conditions beneath discrete deposits. Oxygen is often an important driving force in corrosion (Hamilton 1985; Hardy and Brown 1984) therefore aerated conditions more closely mimic the natural environment.

Corrosion may be studied using electrochemical techniques, by measuring the weight loss of metal specimens and by making qualitative observations of the surface characteristics of the corroded metal (Champion 1965). When studying microbial corrosion, it is important to look for significant localized or pitting corrosion (not just overall corrosion, determined by weight loss) to indicate that biological corrosion has taken place.

B. CLASSIFICATION OF GRAM-NEGATIVE ENVIRONMENTAL ISOLATES

In a series of studies (Obuekwe et al. 1981b; 1981c; 1981d; 1983) on the microbial corrosion of metals by gram-negative, iron-reducing, sulfide-generating bacteria obtained from oil field fluids, such isolates were classified as being members of the genus *Pseudomonas* (C.O. Obuekwe, Ph.D. thesis). However, one isolate, designated Ps 200, had a mol% G+C content of 42 mol% which was lower that which defines the genus *Pseudomonas* (58-70 mol%) and thus the classification of such isolates required further investigation.

Gram-negative, aerobic eubacteria are readily isolated from most aquatic and terrestrial habitats including fluids produced from oil field activities. The evolutionary relationships and the identification and classification of such bacteria have been intensively investigated in the last two decades (Baumann et al. 1972; Baumann and

Baumann 1981; Mandel 1969; Stanier 1971). The information obtained has resulted in the establishment, in Bergey's Manual of Systematic Bacteriology (Baumann et al.1984), of a new genus named *Alteromonas*, although it has not been assigned to a family. Members of this genus have the following characteristics: cells are motile, have polar flagella, oxygen is used as a universal electron acceptor, a seawater base is required for growth and they contain DNA with a G+C content in the range of 38-50 mol% (T_m,Bd). This latter characteristic clearly separates members of this genus from members of the genus *Pseudomonas*.

Lee et al. (1977) carried out a numerical taxonomic study of 26 marine and 12 other *Pseudomonas*-like bacteria, using biochemical tests and G+C content. Included in this survey were strains of *Pseudomonas putrefaciens* isolated from seawater, fish and dairy foods and *Pseudomonas rubescens* isolated from cutting oil (Pivnick 1955). The isolates were grouped into five phenons - two with greater than 55 mol% G+C were regarded as *Pseudomonas* spp. and three with G+C content between 43 - 55 mol% G+C were grouped as *Alteromonas* spp. It was concluded that the isolates of Derby and Hammer (1931), and many of those isolated from the sea and fish which were previously classified as *P. putrefaciens* (Long and Hammer 1941) and *P. rubescens* (Pivnick 1955), would be more suitably classified as *Alteromonas putrefaciens*. This species, however, is placed in the category "species incertae sedis" and appended to the description of the genus *Alteromonas* in the 1984 edition of Bergey's Manual of Systematic Bacteriology.

The minimum chartacteristics for identification of an isolate as a strain of *A. putrefaciens* include: a gram-negative reaction, polar flagella, an oxidase positive reaction, production of DNase and ornithine decarboxylase, and the ability to produce hydrogen sulfide (Hugh and Gilardi 1980). Two distinct groupings of this species have been reported: one unable to grow in 7.5% NaCl but which grows at 4°C and another with a higher G+C content which is salt tolerant but unable to grow at 4°C (Riley et al. 1972; Levin 1972; Holmes et al. 1975; Owen et al. 1978).

Isolates of *A. putrefaciens* are also distinguished from other pseudomonads by their ability to reduce trimethylamine oxide (TMAO) (Hendrie and Shewan 1979), a major reaction during anaerobic fish spoilage, in which TMAO functions as a terminal electron acceptor during anaerobic respiration (Ringo et al. 1984). Bacterial TMAO reduction has been reviewed by Barrett and Kwan (1985). This ability was thought to be wide spread among bacterial genera, but is limited to some *Vibrio* spp., Enterobacteriaceae, and *Alteromonas* spp. as a result of the reclassification of *P. putrefaciens* to the genus *Alteromonas*. Strains of marine *A. putrefaciens* do not require seawater base for growth but studies of Stenberg et al. (1984) have shown sodium was required for anaerobic active transport of certain amino acids during TMAO reduction.

Strains of *A. putrefaciens* are not only important in the low temperature spoiling of marine fish and dairy products (Shewan 1974), and as clinical isolates (Hugh and Gilardi 1980) but are widely distributed in soils and water (Hugh and Gilardi 1980). They have also been identified as components of marine microfouling films (Zambon et al. 1984). Strains have been isolated from machine cutting oil emulsions (Pivnick 1955) and aluminum rolling mill coolants (Hill 1976). Microbial deterioration of machine oil emulsions and corrosion of machine parts has been reviewed by Hill (1971) and Genner and Hill (1981). Iisuka and Komogata (1964) isolated two strains from petroleum brines in Japan. Strains of these organisms are also widely distributed in oil field fluids produced in north central Alberta (C.O. Obuekwe, Ph.D. thesis; Westlake et al. 1986; Semple and Westlake 1987a).

C. IRON-REDUCING BACTERIA

Iron reduction and the iron cycle have been discussed by Ehrlich (1981) and Nealson (1983). Iron-reducing bacteria are ubiquitous in soil, groundwater, lakes and marine environments and are studied because of their importance in iron cycling and making iron biologically available in these environments. These organisms have the

ability to reduce ferric iron, often found in nature in the form of amorphous or crystalline insoluble oxides thereby releasing the more soluble and mobile ferrous ions. Microbial mineral transformation is important in agricultural soils because it affects mineral availability, soil fertility and soil formation.

Ferrous iron release is due to the direct action of iron-reducing bacteria, coupled with substrate oxidation. Iron reduction has been found to be a major pathway in the anaerobic breakdown of organic matter in anoxic lakes (Jones et al. 1983; 1984b), marine and estuarine sediments (Sorenson 1982; Tugel et al. 1986; Lovley and Phillips 1986a,1986b) and soils (Kamura et al. 1963; Munch and Ottow 1983). Welp and Bruemmer (1985) used an iron reduction test as a measure of the effects of toxic chemicals on microbial activity in soils. Reduction of insoluble ferric oxides requires direct cell contact (Tugel et al. 1986; Munch and Ottow 1982,1983; Arnold et al. 1986b) and the oxide form influences the reducing ability with amorphous iron oxides being preferentially reduced to crystalline forms (Munch and Ottow 1980,1982; Lovley and Phillips 1986a). Iron speciation, that is, the presence of iron bound to specific ligands, also affected the rate of iron reduction by isolate Ps 200 (Arnold et al. 1986a, 1986b).

The ability to reduce ferric iron has been reported for a diverse group of organisms (Ottow 1969b; Ottow and Glathe 1971; Jones et al. 1984a), many of which are facultative nitrate-reducing bacteria. Nitrate reductase may have iron reductase activity in some organisms but there is much evidence for the existence of specific iron reductase systems (Hamman and Ottow 1974; Ottow and Glathe 1971; Ottow and Munch 1978; Munch and Ottow 1983). In addition, nitrate reductase positive organisms which do not reduce iron, and iron-reducers which do not reduce nitrate, have been isolated (Pfanneberg and Fischer 1984; Jones et al. 1984a). Autotrophic iron-reducers, which use H_2 for energy, have been isolated by Jones et al. (1983) and Balashova et al. (1980). Iron reduction has been studied in sulfur- and iron-oxidizing bacteria like *Thiobacillus ferrooxidans* (Brock and Gustafson 1976; Sugio et al. 1984). No other groups have reported iron reduction

by *A. putrefaciens* strains and Arnold et al. (1986 a) reported that isolate Ps 200 reduced ferric iron at considerably higher rates than have been reported for other bacteria.

The characteristics of dissimilative iron reduction in isolate Ps 200 were extensively studied by Obuekwe (Ph.D. thesis). This organism has a cell-associated, iron reductase system which is induced in iron-containing medium, is linked to electron transport and whose activity is related to cytochrome levels in the cell (Obuekwe and Westlake 1982a). Inhibition experiments suggested a model of electron transport to Fe(III) via cytochrome b (Obuekwe et al. 1981a). The relationship between nitrate and iron reduction was explored by Obuekwe (Obuekwe et al. 1981a; Obuekwe and Westlake 1982a,1982b), who showed that iron reduction was enhanced for a short time in nitrate-induced cells and that nitrate did not inhibit iron reduction until nitrate reductase had been induced. Recently, Arnold et al. (1986a) reported the existence of a constitutive and an inducible iron reductase in isolate Ps 200, which they temporarily named "*P. ferrireductans*". The inducible ferrireductase was formed under low oxygen conditions and was capable of iron reduction at rates one order of magnitude faster than the constitutive form. They also reported that constitutive iron reduction was coupled to oxidative phosphorylation whereas induced reduction was not. Models proposed by Arnold (1986a, 1986b) for the electron-transport chain configuration to ferrireductase are similar to those found for *A. putrefaciens* from studies of TMAO reduction (Easter et al. 1983; Stenberg et al. 1984).

D. SULFITE REDUCTION AND SULFUR ISOTOPE FRACTIONATION

Cultures of *A. putrefaciens* are characterized by their ability to produce hydrogen sulfide (Hendrie and Shewan 1979; Hugh and Gilardi 1980). Of the commonly isolated sulfide-generating bacteria, facultative, fermentative strains include of the genera *Proteus*, *Citrobacter* and *Salmonella* (McMeekin and Patterson 1975; Oltmann et al. 1975). *A. putrefaciens* strains are the only non-fermentative organisms that produce hydrogen sulfide (Hugh and Gilardi 1980). However, little information is available on the

characteristics of their sulfide-generating systems. Some strains reduce both sulfite and thiosulfate to sulfide while others produce sulfide only from thiosulfate (Semple and Westlake 1987a). Obuekwe et al. (1983) studied the interaction of sulfite- and thiosulfate-reducing strains and proposed a "cascade of sulfide generation". Such organisms could contribute to the overall sulfide generation capacity present in crude oil deposits, in pipelines and in storage tanks.

Laishley and Krouse (1978) clearly established the presence of a dissimilatory sulfite reduction process in *Clostridium pasteurianum* and recently showed that it is present in five other clostridial species (Laishley et al. 1984). They hypothesize that the ability to carry out dissimilatory sulfite reduction is probably a characteristic of many clostridial species. These results clearly indicate that dissimilatory sulfite reduction is a process which is not restricted solely to the classical sulfate-reducers, that is, members of the genera *Desulfovibrio* and *Desulfotomaculum*.

Advances in the field of bacterial sulfur isotope fractionation have been reviewed by Chambers and Trudinger (1979). Sulfur isotope fractionation has been associated with microbial dissimilatory sulfate reduction (Harrison and Thode 1958; Kaplan and Rittenberg 1964) and has recently been studied with dissimilatory sulfite reduction in *Clostridium* (McCready et al. 1975; Laishley and Krouse 1978). Isotope fractionation studies have also been carried out with fermentative organisms such as *Proteus vulgaris* (McCready et al. 1980) and *Salmonella heidelberg* (McCready and Krouse 1979).

The purpose of sulfur isotope fractionation studies is to define those reaction which alter the sulfur isotope abundance in natural situations and to study mechanisms of microbiological sulfur transformation reactions by examining the isotope fractionations realized (McCready et al. 1975). Complex isotope fractionation patterns with normal and "inverse" kinetic isotope effects (where the evolved sulfur is enriched in ^{34}S in comparison to the reservoir sulfur) have been reported during sulfite reduction in the studies with *Clostridium* spp. (Laishley et al. 1976, 1984; McCready et al. 1975) and

Salmonella sp. (McCready and Krouse 1979; McCready et al. 1980). These patterns are predictable and have been used to diagnose the presence of dissimilatory sulfite reductase (Laishley et al. 1984). The objective of these studies was to generate sulfide in a closed reaction system and to let the reaction go to completion. Collection of the sulfide at intervals as it is produced and monitoring the instantaneous isotope composition of the reaction product, rather than combining it all, generates complex isotope fractionation patterns. Isotope fractionation in closed systems has been discussed by Rees (1973) and various explanations for the inverse isotope fractionation patterns have been proposed by Krouse's group (McCready et al. 1975; Laishley et al. 1976; McCready and Krouse 1979).

Sulfur isotope fractionation studies were initiated to test the ability of oil field isolates, including isolate Ps 200, to grow anaerobically on sulfite, to measure the sulfur isotope fractionation during sulfite reduction and to compare the isotope patterns to those of organisms reported in the literature. The report presented by Semple and Westlake (1987b) describes the sulfur isotope fractionation patterns observed during sulfite reduction by four strains of *A. putrefaciens* isolated from oil field fluids.

E. SUMMARY

Microorganisms have had a major impact on corrosion in the oil industry. Most of the literature describes corrosion by SRB but it is becoming recognized that there are other organisms important in the corrosion process which may act alone or in concert with the SRB. From this review, it is evident that more information is needed on the bacteria which utilize intermediates in the sulfur cycle and may be involved in generating H_2S, as weel as those which reduce soluble ferric oxide films and affect the concentration of soluble iron. The effects of these organisms on corrosion by SRB in mixed culture should be initiated to better mimic real life corrosion situations. Investigation into the

distribution and characteristics of these facultative aerobic bacteria is important for the understanding of the influence of microbes on the corrosion process and their involvement in biogeochemical cycles for iron and sulfur.

III MATERIALS AND METHODS

A. SOURCES OF OIL FIELD SAMPLES

Oil from various producing oil fields in central and northern Alberta is shipped by pipeline to the Interprovincial Pipe Line (IPL) terminal at Edmonton. The oil is stored in large capacity tanks, 22029 m^3 to 45807 m^3, for shipment to refineries in eastern Canada and the USA. The water phase which separates from the oil during storage should contain microbial populations representative of possible corrosion-causing organisms existing in the oil fields. Oil storage tanks from 7 oil fields (Table 1) were sampled during 7 sample trips over a 15 month period from May 1984 to November 1985. Draw-off water samples were collected in sterile, methylene-chloride-rinsed 4 litre Nalgene bottles from a valve at the bottom of each tank. The valve was opened and allowed to run for about one minute to remove sludge from the line and to obtain a sample representing the water in the bottom of the tanks. The water temperature was measured on site, on a sample collected in a separate container.

Oil samples from 5 of the oil fields (Pembina, Federated, Cold Lake, Wizard Lake and Ellerslie) were collected from the inflowing oil pipelines in sterile 250 ml bottles. Produced water samples also were obtained from 10 producing wells in the Esso Norbuck Field, Drayton Valley, Alberta in November 1985.

All bottles were filled, transported to the lab, stored at 4°C and analyzed within 24 hours after collection. Salinity measurements were made on the aqueous phase of samples using a YSI Model 33 salinity meter (Yellow Springs Instrument Co). Samples with salinity greater than 40 parts per thousand (ppt) were diluted into a readable range by the addition of distilled water. The pH of the samples was determined with a Fisher Accumet pH meter.

Table 1. Summary of oil storage tanks sampled at Interprovincial Pipe Line (IPL) terminal, Edmonton, Alberta, Canada

Tank #	Oil field serviced	Tank capacity (m^3)	
		Rated capacity	Working capacity
2	Rainbow Lake	22029	18611
5	Wizard Lake-Ellerslie	22029	18611
18	Cold Lake	23876	19845
19	Federated (Swan Hills)	23886	20140
23	Pembina	34314	28147
24	Redwater Mix Blend	45807	38197

B. ENUMERATION AND ISOLATION OF MICROORGANISMS

Previous studies have shown that salt was required in media for maximal recovery of microbial populations from highly saline oil field water samples. For example, statistically higher bacterial counts were obtained from Judy Creek oil field samples on media containing 4.5 to 7.0 % NaCl (Westlake, Ward and Cook. 1979. University of Alberta, Edmonton, Unpublished data). All oil field waters sampled in the present studies were saline, but with salinities ranging from 8 to 120 ppt. Based on a comparison of plate counts and Most Probable Number (MPN) determinations in media containing 0, 2.5, and 4.5 % NaCl, the optimum salt concentration for maximal recovery of bacteria was found to be 2.5 % NaCl. This involved three oil field water samples with salinities of 24, 54 and 86 ppt and in all cases, counts determined on media containing 2.5 % NaCl were either significantly higher or no different from counts measured without salt or with 4.5 % NaCl. All media and dilution blanks were subsequently prepared with 2.5 % (i.e. 25 g/l) NaCl.

Samples were mixed by shaking vigorously 30 times before aliquots were taken for microbial analysis. For water samples containing oil, water for analysis was taken from below the oil layer. A 10-fold dilution series was made by measuring 10 ml of sample into 90 ml dilution blanks of 0.03 M potassium phosphate buffer (pH 7.2).

Total aerobic bacteria

Aerobic viable cell numbers were determined by spreading 0.1 ml aliquots, 5 plates per dilution, onto Plate Count Agar (PCA: Difco). Oil samples were plated directly, without dilution. Plates were incubated at approximately 23 °C for 5 days, when total and differential counts were carried out based on colonial morphology and pigmentation.

Sulfide-generating bacteria

The number of sulfide-generating bacteria, including sulfate- and sulfite-reducing

bacteria, was determined by a 5 tube/dilution Most Probable Number (MPN) method. Kaput-capped (Bellco) 18x150 mm test tubes containing 10 ml modified Butlin's medium (Butlin et al. 1949: Appendix A) were prepared on the day before use. Iron finishing nails (2 cm) were added to each tube to reduce the medium and all tubes were sterilized by autoclaving for 20 min at 121°C. Filter-sterilized 10% Na_2SO_3 (0.1 ml or 3 drops with Pasteur pipette) was added to each MPN tube just prior to inoculation with 1 ml of sample. Sulfate-reducing bacteria (SRB) were enumerated using the same method without the addition of sulfite.

All tubes were incubated at 23°C and scored weekly for blackening of the nails and surrounding medium which indicates the production of sulfides. The most probable number of sulfide-generating organisms and SRB was calculated from the distribution of positive tubes after 28 days by referring to statistical tables (Alexander 1965). Statistical comparison of MPN values was done using the method of Cochran (1950).

Iron-reducing bacteria

Growth of iron-reducing bacteria in ferric iron-rich B10 broth medium (Obuekwe et al. 1981a,b: Appendix A) results in a color change of the medium from brownish-gold to green as ferrous ions are produced (Figure 4a). This reaction was used to determine the total number of iron-reducing bacteria (including aerobes and anaerobes) by the MPN method. Tubes of B10 broth or soft agar were inoculated with 1 ml aliquots from the dilution series (5 tubes per dilution) and incubated for 7 days at room temperature. Tubes were scored for growth and color change to green or white, and the most probable number was calculated from statistical tables (Alexander 1965).

Some aerobic iron-reducing bacteria, including those isolated by Obuekwe (Ph.D. thesis), form concave, orange colonies (Figure 4b) when grown on B10 agar plates (C. Panter. 1968. M.Sc. thesis, University of Alberta, Edmonton, Alberta: Appendix A).

Figure 4. Growth of iron-reducing bacterium Ps 200 in (A) B10 broth and (B) on B10 agar plates.

A. Note the color of the control changes from gold (1) to green (2) with the production of ferrous ions. Some tubes further change to a whitish color (3).

B. Concave colonies of isolate Ps 200 on the surface of an agar plate. Note the orange color of the colonies and the depressed centres (i.e. cross section = ⌒)

A

1 2 3

B

Viable plate counts of these aerobic iron-reducing bacteria were determined by plating aliquots from the dilution series, 5 plates per dilution, and incubating at 23°C for 5 days. Total and detailed counts of the number of different colonial morphologies were determined, including the orange-colored, concave colonies. Representative concave, orange colonies from B10 plates were selected and purified by repeated plating onto the same medium. All purified isolates were transferred into B10 broth to confirm their iron-reducing abilities as it was observed that not all concave colonies would reduce ferric iron. Some of these iron-reducing bacteria were shown to produce characteristic pink to orange pigmented colonies on PCA, so selected pigmented colonies from PCA plates also were purified and tested for their ability to reduce iron.

Anaerobic isolation of iron-reducing bacteria was carried out on samples from positive MPN tubes, which were plated onto B10 agar plates and incubated in Gas Pak (BBL) anaerobic jars. The bacteria growing on anaerobic plates showed different colonial morphologies, so representative colony types were replated onto duplicate B10 plates. One plate was again incubated anaerobically and the other was exposed to air to test for aerobic growth and the formation of concave colonies. These isolates also were grown in B10 broth to reconfirm their ability to reduce ferric iron.

Maintenance of the culture collection

The culture collection of 80 iron-reducing isolates was maintained at 4°C by monthly transfers into tubes of B10 soft agar (0.25% agar). Included in this collection are four strains (Ps 200, 213, 216 and 230) isolated by Obuekwe (Ph.D. thesis) from oil and corroding pipe as described by Obuekwe et al. (1981a, 1983). Isolates were coded using an abbreviation of the oil field from which they were isolated and for some, the trip number.

C. IDENTIFICATION OF IRON-REDUCING BACTERIA

Iron-reducing bacteria isolated from oil field sources were classified using various biochemical and morphological tests which confirmed their identification as strains of *Alteromonas putrefaciens* (Semple and Westlake 1987a). Isolates of *A. putrefaciens*, including ATCC 8071 (type strain), 8072 and 8073 which were isolated from butter (Derby and Hammer 1931) and *'P. rubescens'* ATCC 12099 and 19857 isolated from machine cutting oil (Pivnick 1955), as well as *Pseudomonas aeruginosa* ATCC 10145 and *Proteus vulgaris* ATCC 13315, were included in this survey for comparative purposes.

Preliminary tests on all isolates followed the taxomomy scheme described by Westlake and Cook (1980). The Gram reaction, catalase and oxidase tests were determined using colonies from a 48 hour PCA plate (Difco). Cell morphology and motility were determined with cells grown for 24 hours in 10 ml tubes of modified Butlin's medium (Appendix A) and the method of Mayfield and Innis (1977: Appendix A) was used to examine the flagellar pattern. Twenty-four hour Butlin's tube cultures also served as inoculum for biochemical tests and all tests were carried out at 23°C in media without added NaCl, unless otherwise stated. The utilization of glucose and lactose was determined using oxidative/fermentative (O/F) agar plates of Board and Holding (1960) medium (Appendix A). Duplicate plates were incubated aerobically and anaerobically in Gas Pak (BBL) anaerobic jars and scored for growth and production of either an acid or alkaline reaction after 1 week. Selected isolates were tested using the API 20E Enterobacteriaceae Identification System (Analab Products, Plainview, N.Y., USA).

Further procedures used for the classification of these isolates are outlined by Hendrie and Shewan (1979) and in the ASM Manual of Methods for General Microbiology (Gerhardt 1981). Extracellular hydrolases were assayed in nutrient broth containing 5% gelatin for gelatin liquification and on 0.2% starch agar plates for starch hydrolysis. DNase activity was determined at 30°C on DNase Test Agar (Difco) with 1%

chloroform-extracted methylgreen (0.5% solution) added to accentuate the cleared zones. Citrate utilization was assessed using Simmons citrate agar plates and urease production determined in Bacto-Urea broth (Difco). Ornithine decarboxylase was tested for in Decarboxylase Base broth (Difco) with 1% added L-ornithine (Calbiochem), overlaid with sterile mineral oil after inoculation (Appendix A). Arginine dihydrolase and lysine decarboxylase activities were determined from the results of the API 20E Identification System.

Nitrate reduction was determined in tubes of semisolid agar containing 1% KNO_3 (Stanier et al. 1966) with nitrite production detected by reaction with alpha-naphthylamine and sulfanilic acid reagents (Appendix A). Trimethlyamine oxide (TMAO: Sigma) reduction to trimethlyamine (TMA) was tested for by the method of Wood and Baird (1943) except that the production of TMA was detected on indicator soaked filter papers suspended from a rubber stopper in a plastic cup (Kontes) (Appendix A). TMA production was also assessed using the Laycock and Reiger (1971) assay.

Sulfide generation

The ability of isolates to reduce sulfite and thiosulfate was tested in defined synthetic medium (Obuekwe et al. 1983: Appendix A). Two iron finishing nails were added to each tube and filter sterilized 10% sodium sulfite or sodium thiosulfate was added (0.1 ml/10 ml tube) just prior to inoculation. Sulfide production was monitored daily for up to 7 days as previously described. Sulfide production was also tested by growing selected isolates and *P. vulgaris* on Triple Sugar Iron (TSI) agar (Difco) slants which contain thiosulfate and organic sulfur.

Crude oil utilization

Selected isolates from different oil fields were screened for their ability to utilize

crude oil, as described by Fedorak and Westlake (1981, 1983, 1984). Norman Wells crude oil (0.2 ml) was added to 200 ml of B+N mineral medium (Appendix A) and inoculated with the growth from a 1 week plate of B10 agar suspended in 0.03 M potassium phoshate buffer (pH 7.2). Uninoculated, oil-containing flasks were included as controls. Flasks were incubated for 21 days at 27°C with shaking on a New Brunswick Scientific Model 11 Gyratory shaker at 200 RPM and were checked for purity by restreaking on B10 plates. Oil was extracted, fractionated and analyzed using gas chromatography (GC) and the GC profiles were compared to the controls to determine changes in the saturate, aromatic (Fedorak and Westlake 1981) and sulfur heterocycle profiles (Fedorak and Westlake 1984).

DNA base composition

The mol% G+C content of selected isolates was determined using the thermal melting point (Tm) method (Johnson 1981). DNA was isolated from 2-3 g of cells grown overnight at 27°C in two 500 ml erlenmeyer flasks with 200 ml modified Butlin's medium, using the Marmur (1961) method. However, only one chloroform-isoamyl alcohol extraction was performed. Ribonucleic acid (RNA) was removed by adding 0.2 mg/ml RNase (Sigma) and incubating for 30 minute incubation while protein was subsequently removed with 0.2 mg/ml pronase (Calbiochem) and a 60 minute incubation, both at 37°C. All prepared DNA was dialysed into 0.5x standard saline citrate (SSC) prior to melting profile determination. Each dialysis batch included a corresponding reference *E. coli* b DNA. The mol% G+C was calculated on the basis of the midpoint temperature of the thermal melting profile (Tm) and compared to the Tm of the *E. coli* b standard (mol% G+C = 51) (Appendix B). A sample of *P. aeruginosa* DNA (mol% G+C = 67) also was prepared for comparative and reference purposes.

Salt tolerance and growth at 4°C

The ability of isolates to grow in the presence of NaCl was tested by incorporating increasing concentrations of NaCl (10, 25, 45, 60 and 75 g/l NaCl) into B10 agar plates. B10 agar plates without NaCl were streaked as a positive control. Growth was determined by visual observation after one week of incubation at room temperature. A B10 plate with no added NaCl was also streaked and incubated for 2 weeks at 4°C to determine the ability of the isolates to grow at a low temperature.

Comparison to salt-requiring *Alteromonas* spp.

The original source and DNA base composition of several pigmented and non-pigmented strains of *Alteromonas* spp. requiring a seawater base for growth are listed in Table 2. These isolates were obtained to compare their characteristics with those of the oil field isolates and *A. putrefaciens* strains. Tests performed on these sodium-requiring strains used media supplemented with 25 g/l NaCl. These cultures were maintained by transferring weekly onto Marine Agar (Difco) plates.

D. IRON REDUCTION ASSAYS

Determination of iron reduction rates of resting cells

The rates of iron reduction for strains of *A. putrefaciens* were compared using an enzyme assay procedure developed by Obuekwe (Ph.D. thesis; Obuekwe and Westlake 1982b). Iron reduction rates were determined using whole, resting cells of late exponential phase culture in a defined assay mixture of lactate and soluble ferric iron at 30°C. Rates expressed were based on total cell protein.

a. Resting cell preparation: Cells were grown in 250 ml flasks containing 100 ml of modified Butlin's medium, using as inoculum 1 ml of cells grown for 24 hour in 10 ml

Table 2. Sources of salt requiring, low mol% G+C *Alteromonas* sp. strains

Name	ATCC no.	Pigment	mol% G+C	Source	References
A. macleodii (Type strain)	27126	none	45.6±0.8	seawater	Baumann et al. 1972
A. haloplanktis	14393	none	43.2±1.0	seawater	Baumann et al. 1972
Ps. atlantica	19262	none	43.5	seaweed	Yaphe 1957; Mandel 1966
A. piscicida	15251	yellow	44.5	red tide bloom	Bein 1954; Mandel et al. 1965; Hansen et al. 1965
A. aurantia	33046	orange	38-43	seawater	Gauthier and Breittmayer 1979
A. rubra	29570	red	46-48	surface seawater	Gauthier 1976

tubes of modified Butlin's medium. Flasks were incubated at 27°C on a New Brunswick Scientific Model 11 Gyratory Shaker running at 200 RPM. Growth of the cultures was followed by taking measurements of the optical density (OD) of 1 ml aliquots of cells at 600 nm using a 1 cm light path. Isolates were grown for 13-15 hours and the OD was measured 2-3 times in the last hour before harvesting to determine the approximate stage of growth. Cells were collected in sterile 250 ml Nalgene centrifuge bottles by centrifugation at 10,000 x g at 4°C for 15 minutes, washed two times with 0.1 M potassium phosphate buffer (pH 7.2) and resuspended at a concentration of 1 g wet weight per 80 ml of buffer. These suspensions were used for determination of iron reduction rates. Resting cells were stored on ice during preparation, then at 4°C to determine the affect of storage time on iron reduction rate.

The protein concentrations of the resting cell suspensions were determined after alkaline digestion using the method of Lowry et al. (1951) with bovine gamma-globulin (Biorad) as a reference standard (Appendix C).

Cell numbers were determined by plating in triplicate onto B10 agar plates using 9 ml of 0.03 M potassium phosphate buffer (pH 7.2) dilution blanks for dilutions. Plates were counted after 3 days incubation at 23°C. B10 agar plates was used because isolate Ps.200 showed significantly higher counts on B10 than on PCA and other isolates did not grow as well on PCA as on B10.

b. Assay conditions: Iron reduction rate assays were carried out in screw cap assay tubes containing 2 ml of 2% soluble ferric phosphate (pH 7.2) (City Chemical Company, New York) and 1 ml of sodium lactate (300 µmol/ml) (Fisher Scientific). Increasing volumes of resting cell suspension were used (0.5, 1, 1.5, 2, 3 ml etc.) with the final volume of 10 ml made up using sterile distilled water. The effects of increasing lactate concentration were tested using different volumes of lactate solution (0.5, 1, 1.5 and 2 ml) and 1 ml of resting cell suspension.

Tubes were incubated in a 30°C water bath. To test for temperature effects on iron reduction rates, tubes incubated at 23°C or in a 37°C water bath were also included. The production of Fe^{2+} was assayed every 30 minutes for 3 hours using the colorimetric ortho-phenanthroline method (Krishna Murti et al. 1966; Greenberg et al. 1985: Appendix D). To avoid oxidation of the Fe^{2+}, 100 µl aliquots of the sample were transferred directly into measured volumes of buffered o-phenanthroline reagent. The absorbance at 510 nm was read on a Pye-Unicam SP8-500 UV-Vis spectrophotometer within 5 minutes, using a blank of the same dilution of uninoculated control. The concentration of Fe^{2+} was calculated by referring to a standard curve (Appendix D). Intervals of 30 minutes allowed sufficient time to dispense the reagents, take samples and read them.

The maximum rate of Fe^{2+} production for each cell volume was calculated and plotted to confirm a linear increase in rate with increase in cell protein concentration. The rate of ferric iron reduction for each isolate was calculated from the Fe^{2+} production over 3 hours based on the protein concentration of the volume of resting cells used. This was expressed as milligrams Fe^{2+} per litre per hour per milligram protein. Iron-reducing activity was then calculated, considering that the total reaction volume was 10 ml, as µmol Fe^{2+} / h / mg protein.

c. Iron reduction by AN13: Isolate coryneform AN13 did not grow well in modified Butlin's medium nor did it reduce ferric iron in the assay designed for *A. putrefaciens* strains. This isolate was grown for 15 hours at 37°C in B10 medium without ferric phosphate added and resting cells prepared as previously described. Iron reduction assays were tested using 1 ml of resting cells with 2 ml of 2% $FePO_4$ and 7 ml of iron free B10 medium at 37°C. Resting cells of isolates ESSO 1-3, Ps 200 and ATCC 8071 also were tested in the same assay for comparative purposes.

Solubilization of insoluble iron oxides

The ability of isolate Ps 200 to release ferrous ions from various amorphous and crystalline iron oxides was tested. Synthetic red iron oxide (Fe_2O_3) and black ferri/ferrous hydroxide (Fe_3O_4) were obtained from Fisher Scientific as fine powders. The other iron minerals used were supplied by Dr. W.J. Page (Department of Microbiology, University of Alberta) and were ground to approximately 200 mesh before use (Page and Huyer 1984).

Screw cap tubes (16x100 mm) containing 50 mg of ground oxide were sterilized twice for 30 minutes at 121°C. The oxides and media were sterilized separately to avoid reduction of oxide by media components (Fischer and Pfanneberg 1984). Ten ml of sterile iron-free B10 medium was added to each tube, then inoculated in triplicate with 0.1 ml of 18 hour Butlin's broth culture. Tubes were sealed, mixed and incubated at 23°C on their sides to expose the maximum amount of the oxide to bacterial attack. Uninoculated controls for each mineral were included to test the solubility of the mineral and tubes of B10 medium with soluble ferric phosphate were included for comparison. The ability of selected isolates and Ps 200 to release ferrous iron from red Fe_2O_3 powder (Fisher) was compared using 10 ml of iron-free Butlin's medium containing lactate as the energy source.

The amount of ferrous iron released was assayed as previously described. Tubes were mixed, and allowed to settle upright before the samples were taken to avoid interference of the powdered mineral. Samples were transferred immediately to the o-phenanthroline reagent to avoid oxidation. Triplicate tubes were opened on the same schedule to compensate for small amounts of aerobic growth that may have occurred after opening, since ferrous iron release was higher in tubes opened periodically during time-course experiments than in unopened tubes. The pH of the tubes was measured at the end of each experiment.

E. SULFUR ISOTOPE FRACTIONATION STUDIES

Organisms

Four isolates, Ps 200, CL41, Fed 3 and Pem 12, were chosen for sulfur isotope fractionation studies. These isolates were from different oil fields and showed rapid production of sulfide (within 2 days) from sulfite in tubes of Butlin's medium. These isolates also represented the two groups of sulfite-reducing *A. putrefaciens* strains; Ps 200 and Pem 12 grew at 4°C but were not salt tolerant whereas Fed 3 and CL 41 had a higher mol% G+C and would grow in the presence of 7.5% NaCl but not at 4°C.

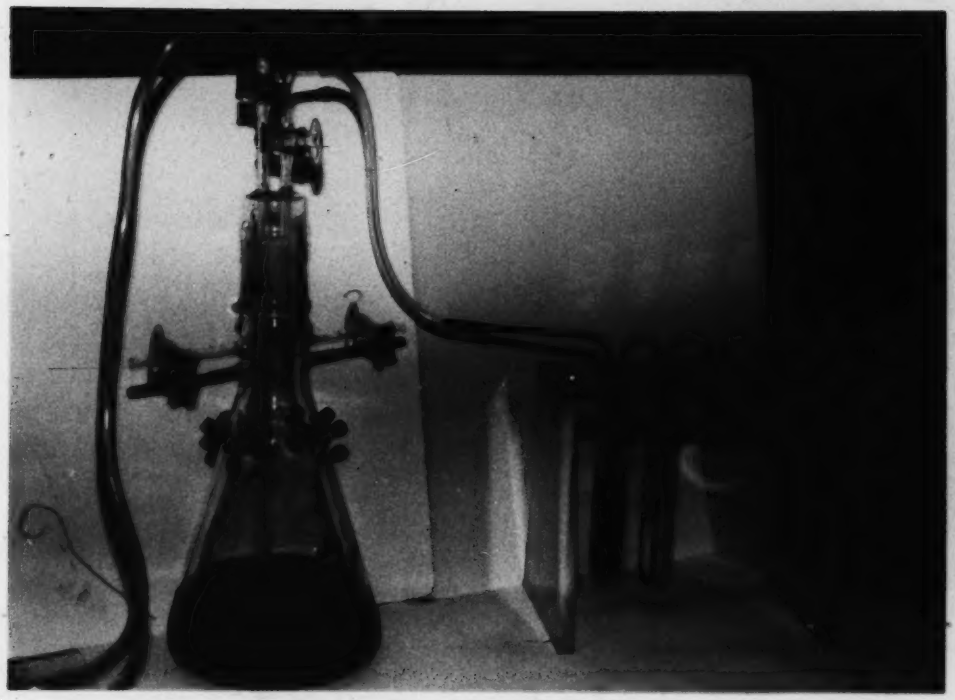
Anaerobic culture system

The culture system used (Figure 5) was a modification of the apparatus described by Krouse et al. (1967). Isolates were grown anaerobically in sealed 2 litre flasks at 23°C in 1 liter of medium (Appendix A) where sulfate constituents were substituted with chlorides and no extra iron was added to reduce FeS formation. Cultures were sparged at a rate of 1.5 l/min with N_2 gas which had been deoxygenated by passing through a heated copper coil (Sargent Welch). Sulfite (7.93 mmol/l) was added as a filter sterilized solution of $Na_2S_2O_3$ (Fisher Scientific) just before inoculating with 10 ml of culture which had been grown aerobically for 16 hours in 100 ml of modified Butlin's medium as previously described. Growth was followed by measuring the optical density of 1 ml aliquots of culture at 600 nm using a 1 cm light path on a Pye-Unicam SP8-500 UV-Vis spectrophotometer. The pH of each aliquot was measured and changes in cell morphology and motility were observed using phase contrast microscopy.

Collection and recovery of sulfide

The H_2S gas produced was collected at intervals by trapping as cadmium sulfide.

Figure 5. Sulfite reduction vessel with cadmium acetate trap.



This was subsequently converted to silver sulfide for gravimetric and stable sulfur isotope analysis as described by Laishley and Krouse (1978), with the following modifications. The hydrogen sulfide was flushed through a trap assembly (Figure 5) which consisted of two 20x150 mm screw cap test tubes in series, sealed with a 20 mm butyl rubber washer with 2 mm teflon tubing as bubblers. The entire assembly was in a plexiglass stand for easy changing of the collectors. Tubes contained 25-30 ml of 10% cadmium acetate buffer (125 g cadmium acetate + 250 ml concentrated acetic acid + 1000 ml distilled H_2O).

Trapping tubes were changed at frequent intervals, when the buffer had turned yellow from the formation of CdS. The solution was transferred to a 400 ml beaker and was converted to Ag_2S by adding 10 ml of 10% $AgNO_3$. In order to raise the pH and prevent formation of a flocculant white precipitate which formed upon addition of silver nitrate to cadmium acetate, more NH_4OH was required than suggested by Laishley and Krouse (1978). Raising the pH to 9 by repeated NH_4OH additions, with vigourous stirring and heat, dissolved the white precipitate and allowed the black Ag_2S to precipitate out of solution.

After the silver sulfide had digested on a steam bath for 1-2 hours, the solution was then filtered through pre-weighed Millipore filters (4.5 cm, Type HA, 0.45 μm). Filters were oven dried at 100°C for 12 hours, cooled and weighed to the nearest 0.1 mg. Sulfide production was calculated from the weight of the silver sulfide precipitate.

Isotope composition of reservoir sulfur

The sulfur isotope composition of the residual sulfur after sulfide generation had ceased, was determined by oxidation of a volume of the medium with hydrogen peroxide and recovery of the sulfate produced as barium sulfate (McCready et al. 1975). About

100 ml of culture was centrifuged at 10,000 x g for 15 minutes to remove the bacteria and the liquid then decanted into a beaker. Hydrogen peroxide (30%) was added, followed by 10 ml of 10% barium chloride. The white $BaSO_4$ precipitate was collected by Millipore filtration (0.45 μm) and oven dried at 100°C. This procedure also was used to determine the $\delta^{34}S$ of the original Na_2SO_3 by adding 1 ml of 10% Na_2SO_3 to 100 ml fresh medium, oxidizing to sulfate with H_2O_2 and collecting the $BaSO_4$ precipitate. No precipitate formed in the medium without the addition of sulfite, as the added sulfite was the only form of sulfur present.

Sulfur isotope fractionation analysis

The silver sulfide was analysed for stable isotope composition as described by McCready et al. (1975) using standard mass spectrometric techniques. The sulfur isotope composition of the sulfide produced is expressed as $\delta^{34}S$ in parts per thousand (‰) with respect to that of the initial sulfite by the formula:

$$\delta^{34}S = \left[\frac{(^{34}S/^{32}S)\ H_2S \text{ product}}{(^{34}S/^{32}S) \text{ initial sulfite}} - 1 \right] X\ 1000$$

$^{34}S/^{32}S$ is the ratio of the numbers of ^{34}S to ^{32}S atoms in the sample as determined by mass spectrometry.

The isotope composition of the remaining sulfur in the reservoir at each time interval was calculated on the basis of the $\delta^{34}S$ of the evolved sulfide according to the formula of McCready and Krouse (1979: Appendix G).

F. CORROSION STUDIES WITH IRON-REDUCING BACTERIA AND SULFATE-REDUCING BACTERIA

These experiments were designed to compare the attachment to and corrosion of mild steel coupons by iron-reducing bacterium Ps 200 in pure and mixed culture with a strain of a sulfate-reducing bacteria, *Desulfovibrio vulgaris* var *oxamicus* (A.M. Jobson. 1975. Ph.D. thesis, University of Alberta, Edmonton, Alberta).

Corrosion coupons

Corrosion coupons made of AISI 10-18 mild steel were obtained from Caproco Corrosion Consultants, Edmonton, Alberta, Canada. Their dimensions were 5.08 x 1.27 x 0.16 cm with two 0.8 cm holes containing Delrin washers to insulate the coupon from the mounting holders. The washer from the lower hole was removed and the top one used in conjunction with holders in the test flasks. The total exposed surface area with only one washer was 11.79 cm^2. Each coupon had a stamped identification number and came sand-blasted, pre-weighed and individually packaged in a protective envelope.

The uniform size and surface preparation of these coupons make them ideal for corrosion testing by weight loss measurements. In addition, observations of corrosion products, type and extent of corrosion and attachment of organisms can be made on these coupons. They have been used by other scientists to monitor bacterial attachment, activity and corrosion in the field (Sanders and Maxwell 1983; Chen and Chen 1984; Dewar 1986; Hamilton and Maxwell 1986) and the laboratory (Mara and Williams 1972; King et al. 1973b; Obuekwe et al. 1981b).

Corrosion test culture media

The culture medium used for these experiments was modified Butlin's medium (Appendix A), which is a lactate-mineral salts medium containing yeast extract with a low

iron concentration and no reducing agents added. All media used in these tests contained 10 g/l NaCl which is close to the salinity of the produced water from which these organisms were isolated. Both isolate Ps 200 and *Desulfovibrio vulgaris* AL1 tolerate and grow well in media with 10 g/l NaCl added. Corrosion tests have previously been carried out in this medium by Obuekwe (Ph.D. thesis), and it is similar to other culture media used such as Postgate B (Gaylarde and Johnston 1980, 1982, 1986) and the lactate-sulfate-yeast extract medium used by Booth et al. (1966) and King et al. (1973). The effect of yeast extract on culture growth, attachment and corrosion was tested in the same lactate-mineral salts medium minus the yeast extract.

For testing the corrosion caused by isolate Ps 200 under sulfide-generating conditions (C.O. Obuekwe, Ph.D. thesis), filter sterilized 10% $Na_2S_2O_3$ (Fisher Scientific) was added separately to sterilized Butlin's medium at a rate of 1 ml per litre.

A total of 22 test flasks were set up according to the schedule of tests and exposure times listed in Table 3. Sterile controls were included for each time point and medium tested. A separate flask was set up for each test condition and time point to ensure that a constant surface area of metal was exposed to each culture (Mara and Williams 1972; Champion 1965). Coupons were exposed to continuous culture for 3, 6 and 12 weeks to generate data for weight loss vs time curves. Short term exposure (2-4 weeks) may yield high corrosion rates but long term exposure (8-12 weeks) is often required to adequately detect and define pitting. Obuekwe (Ph.D. thesis) observed the attachment of one of the test organisms, isolate Ps 200, after 2 weeks and noticed pitting of the coupons under sulfide-generating conditions after 9 weeks.

Continuous culture system

A continuous culture system was developed to study corrosion of mild steel coupons in cultures of bacteria under flowing conditions, such as those found in

Table 3. Corrosion tests schedule

Test medium and Inoculum	Exposure time (weeks) 3	6	12
Butlin's medium			
Ps 200	+	+	+
AL1	nd [a.]	+	+
Mixed	nd	+	+
Sterile Control	+	+	+
Butlin's medium +$S_2O_3^=$			
Ps 200	+	+	+
AL1	nd	nd	+
Mixed	nd	nd	+
Sterile Control	+	+	+
Butlin's without Yeast Extract (-YE)			
Ps 200	nd	nd	+
AL1	nd	nd	+
Mixed	nd	nd	+
Sterile Control	nd	nd	+

[a.] nd= not done

pipelines.

a. Design of continuous culture system: Cultures were grown in continuous culture in 500 ml side-arm Erlenmeyer flasks with media flow fed from large (22 litres) media reservoirs (Figure 6). Each reservoir had a capped, ground glass (T14/35) supply tube through which media was replenished and an air equilibration tube with two Millex FG 50, 0.2 µm filters (Millipore) in series. A separate feed line from the bottom of the reservoir supplied each flask with medium and a constant flow was maintained at a dilution rate of 0.012 hr^{-1} (C.O. Obuekwe, Ph.D. thesis) by calibrated LKB peristaltic pumps. Pump flow rate was set at 6.2 ml/h. The average liquid volume of the flasks was 486±6 ml which resulted in a residence time for the cultures of 3.3 days.

Silicon tubing was used for all feed lines, with glass tubing connections between the large tubing and the LKB silicon tubing (tube size=1 mm). A long length of LKB tubing was used in each pump so that it could be moved every 3 weeks to avoid wear. A flow breaker, held upright by clamps, was placed in the line immediately before each flask to prevent bacterial growth in the line from contaminating the reservoir.

No attempt at aeration or deaeration of the media was made, but cultures were stirred to ensure mixing. Each flask contained a 5 cm teflon-coated magnetic stir bar and was stirred slowly by Fisher Thermix stirrers which do not heat up with extended use.

A #7 rubber stopper coupon holder assembly was made for each flask (Figure 7). Two bent 4 mm glass rods pushed through the stopper provided a solid, inert support for the coupons. With this setup, 4 coupons can be suspended in the middle of the flask and will not move while being stirred. Coupons threaded through the top Delrin washer also are adequately separated on the holder. The glass rods were bent to carry 2 coupons per holder and adjusted (maximum 2.8 mm) so that 2 holders would fit through the mouth and be situated in the center of a 500 ml flask. Holders 9.5 cm long held the coupons vertically 3 cm above the stirbar and 3 cm below the liquid surface.

Figure 6. Experimental set-up for corrosion tests in continuous culture. Each medium reservoir (R) had separate feed lines for 3 or 4 flasks with constant flow being maintained by LKB peristaltic pumps.

R

Figure 7. Holder assembly for corrosion coupons. Note (A) the flow breaker above the feed tube which prevented contamination of the feed lines, and (B) the air equilibration tube with air filter which helped provide constant media flow.

A

B

A 7mm glass feed tube was suspended above the level of the medium to avoid the problem of growth of the bacteria in the feed tube, as was observed with tubes submerged into the medium. At slow flow rates, and with slow stirring, fresh medium dripping from a tube above the growing culture was adequately mixed and dispersed throughout the culture. In order to observe the mixing and flow patterns, methylene-blue-colored water was dripped into flasks containing clear water. An air equilibration tube with a 25 mm Millex FG 0.2 µm filter (Millipore) was required to maintain adequate draining.

b. Assembly and inoculation: Test flasks containing 450 ml of medium were autoclaved for 30 minutes at 121°C. They were prepared the day of use and once cooled, were inoculated with 10 ml of the appropriate organism. Inoculum of isolate Ps 200 was grown overnight in modified Butlin's medium as previously described and *D. vulgaris* AL1 was grown for 48 hours in 30x150 mm screw cap tubes containing 25 ml of modified Butlin's medium and 4 iron finishing nails.

The stopper and holder assemblies, with flow breaker attached, were sterilized separately in pipette bags. Reservoirs containing 15 litres of medium were sterilized for 95 minutes at 121°C.

Coupons were sterilized by immersion for 15 minutes each, first in 70% ethanol, then 95% ethanol. They were dried under a stream of filter-sterilized air, followed by 10 minutes of exposure to UV light (254 nm) in a sterile hood (Canadian Cabinet Co. Ltd.). Coupon numbers, weights and the corresponding tests were recorded. Immediately prior to assembly, the sterilized coupons were slipped onto their holders, with identification stamps facing inward, and placed in the inoculated flasks, which were then sealed with aluminum foil.

All flasks were left overnight, unstirred, to allow the SRB to grow without introducing oxygen. The feed system was assembled by attaching feed lines to the flow breaker above each flask. After 24-36 hours, when culture growth was established,

media flow and stirring were started. Effluent lines were unclamped and the overflow collected in sterile 4 litre glass bottles containing 10% NaOH to trap hydrogen sulfide.

Evaluation of corrosion tests

a. Test medium: Observations of growth in the flasks, development of biofouling on the coupons and changes in corrosion products were made daily and photographs were taken periodically. At the end of the test period, the coupons were removed and the pH and cell counts of the liquid portion of the culture were determined. The viable number of aerobic bacteria in the inoculum and corrosion flasks was monitored using the spread plate method on PCA, as previously described, with counts made after 3 days of incubation at 23°C.

The five-tube MPN method was modified for enumeration of pure cultures of SRB in the inoculum and test flasks. Butlin's medium was dispensed into 16x150 mm screwcap test tubes containing iron nails. Inoculated tubes were each immediately plugged with a sterile, non-absorbant cotton plug, then a non-sterile plug. The top plug was impregnated with 10 drops of 40% pyrogallol followed by 10 drops of saturated sodium carbonate to remove residual oxygen from the headspace (Pankhurst 1971). The extra precaution of adding the pyrogallol plugs was necessary in order to obtain maximum numbers of SRB in pure cultures, whereas the Kaput-capped tubes were adequate for SRB determinations in oil field and other environmental samples (Fedorak et al. 1987). Control and corrosion flasks containing pure cultures of *D. vulgaris* AL1 were streaked on PCA to test for contamination by aerobic bacteria.

b. Corrosion coupons: Coupons were examined after removal from culture and before cleaning, and the color, distribution and type of corrosion products and surface coating was noted. A detailed record was kept of all macroscopic and microscopic

observations made during the cleaning procedure and photographs were taken periodically.

Biofouling and corrosion products were removed from the coupons by sequential washing, sonication and acid treatment. The coupons on the holder were rinsed by repeatedly dipping and swirling in a 1 l beaker of distilled water. Gentle rinsing removed the loosely attached material but this treatment was not standardized. The washer was removed and bent glass rod hooks were used to hold the coupons during the remaining cleaning procedures.

A single rinsed coupons was prepared for scanning electron microscopic (SEM) examination of the surface coating and the attachment of the microorganisms to the coupons. One of the four coupons was fixed in 0.5% glutaraldehyde for 5 minutes and then dehydrated for 5 minutes in each of 30, 50, 70 and 95% ethanol (Obuekwe et al. 1981b). It was then critical-point dried in CO_2 and sputter-coated with 15 nm of gold for observation with a Spectroscan Scanning Electron Microscope. Photographs were taken at various magnifications, from 10 to 4000 times and a size bar included at the bottom of each picture for reference. Elemental analysis of the coupon coating was made using an energy dispersive X-ray diffraction system. The weight of fixed coupons was determined to estimate the amount of corrosion product attached. A weight gain indicated that the corrosion products remained attached to the metal surface.

The remaining 3 coupons were cleaned with a combination of mechanical and chemical cleaning methods recommended by the coupon supplier, Champion (1965) and the National Association of Corrosion Engineers (NACE 1975b; NACE 1984). Ultrasonic cleaning is used for removal of corrosion product and biological material with minimal handling of the coupons. This also allows for differential removal of loosely attached material and tightly bound, adherent coating (Fontana and Greene 1978). In addition, sonication has been used for determination of the numbers of bacteria attached to the metal surface (Sanders and Maxwell 1983).

Individual coupons were placed in 18x150 mm test tubes containing 10 ml of 0.03 M potassium phosphate buffer (pH 7.2) and these were then put in a Bronsonic 42 sonic bath for 1 minute. If substantial coating remained on the coupons, they were transferred to a second tube of fresh buffer for another minute of sonication. Coupons were examined and the material and corrosion products which had been removed were kept for microscopic observation. Although viable cells were often observed, no cell counts were made because the long sonication times used may have caused disruption of cells. Sonicated coupons were rinsed with acetone, dried and weighed to measure the effectiveness of the cleaning procedure. One coupon was fixed and dried for SEM, as previously described, except this coupon was not gold-coated as weight loss measurements were to be taken. The type and distribution of deposits remaining after sonication were evaluated and the coupon surface was examined for corrosion. Partial removal of the biofouling gave an indication of the type of corrosion that had occurred under the film.

Treatment with an 'inhibited' acid is recommended for removal of all residual corrosion products for determination of weight loss due to corrosion and examination of the cleaned metal surface for evidence of corrosion (Champion 1965). The coupons were cleaned with Clarke's solution: 100 ml 1.16 s.g. (80%) HCl + 2 g antimonous oxide + 2 g stannous chloride (Champion 1965). The strong acid removes any corrosion products from the surface while the antimony inhibits attack of metal by forming a thin film over the metal surface and the stannous chloride reduces any corrosive ferric chloride (Clarke 1936). The coupons were immersed for 1 minute then rinsed with saturated sodium bicarbonate, distilled water and ethanol and dried with acetone (NACE 1975b). The weight of each was determined to the nearest 0.1 mg. An untreated control coupon was included to determine weight loss due to acid treatment. Visual and microscopic examination of the surface were made to determine the type and extent of corrosion attack as described by Champion (1965) and NACE (1984). Acid-cleaned coupons were also

examined under the SEM for changes in surface structures in corroded vs. uncorroded specimens.

IV RESULTS AND DISCUSSION

A. ENUMERATION AND ISOLATION OF MICROORGANISMS FROM OIL FIELD SAMPLES

Evaluation of counting methods

Iron-reducing bacteria (C.O. Obuekwe, Ph.D. thesis) were easily recognizable by their concave colonial morphology and orange color when grown on B10 agar and this was used to estimate their incidence in a population. B10 medium was used as a differential medium and is not selective for iron-reducers but may be too rich for some members of the indigenous bacterial population. This is supported by the observation that the viable counts on PCA were higher for most samples than counts on B10 plates; therefore counts on PCA were taken to represent the total aerobic population. However, there was no significant difference between the number of iron-reducing bacteria on B10 medium (concave colonies) or on PCA (orange colonies).

B10 agar is good for detection of iron-reducers but has limitations for enumeration because not all concave colonies on B10 agar are capable of reducing ferric iron. Simply counting concave colonies did not give the true population numbers in some cases. For example, ESSO Lindale and Breton samples yielded many yellowish, dry-surfaced, 'volcano'-shaped colonies with depressed centres which did not reduce iron in B10 broth, and were subsequently identified as being predominantly *Pseudomonas* spp. or *Bacillus* spp. Upon further purification and replating on B10, many of these isolates did not reform concave colonies. This emphasizes the importance of testing representative colonies to confirm their iron-reducing ability in B10 broth.

The MPN method for enumerating iron-reducing bacteria yielded higher counts than aerobic plate counts for some Pembina and Cold Lake samples. This was because the broth or soft agar tubes allowed for detection of the total number of iron-reducers,

including those aerobic bacteria that did not form concave colonies (C. Panter, M.Sc. thesis) and some of the iron-reducing, anaerobic clostridia. In all instances, aerobic, iron-reducing bacteria comprised a substantial portion of the total iron-reducing population detected.

B10 broth, however, was not always reliable for MPN determination. Interfering reactions sometimes masked the determination of the yellow to green color change. These included blackening caused by the presence of sulfide and precipitation of the medium components because of the saline water and by the activities of unknown bacteria. This was, in part, avoided by using B10 soft agar tubes, in which precipitation was prevented and individual black, green or white colonies could form. Extensive blackening of the tubes still occurred in the tubes from lower dilutions, either because of the sulfide present in the water samples or sulfide production by SRB. In conclusion, it is recommended that both the plate count and MPN methods for detecting and enumerating iron-reducing bacteria should be used in conjunction, to determine the presence of these bacteria in oil field samples.

Distribution of bacteria in oil field samples

The average pH and salinity, the number of samples analyzed and the number of different strains of iron-reducing bacteria isolated and characterized from each source is presented in Table 4. The average pH of the sampled water ranged from 5.5 to 7.2 and the salinity ranged from 8 to 120 parts per thousand (ppt).

The sample temperatures and the total number of aerobic, sulfide-generating and iron-reducing bacteria detected in the IPL draw-off waters are shown in Table 5. The number of aerobes and sulfide-generating bacteria varied in a random manner, both with the source of sample and the time of sampling. Seasonal variations were observed with some samples, such as Pembina and Federated, with a tendency towards higher counts in

Table 4. Summary of oil fields sampled for iron-reducing bacteria

Oil field sampled	Abbreviation	Number of samples analyzed	Salinity (ppt)	pH	Number of different iron-reducing strains isolated
IPL Tank					
Cold Lake 18	CL	4	8±0.5	6.1±0.2	41
Pembina 23	PEM	5	20±6	6.4±0.2	8
Federated 19	FED	5	52±13	6.3±0.3	19
Redwater 24	RED	2	71±21	6.4±0.1	4
Wizard Lake 5	-	1	120	6.5	0
Rainbow Lake 2	-	1	90	5.5	0
ESSO Norbuck					
Breton	ESSO	5	10.5±1.9	7.2±0.1	8
Lindale	-	5	-	-	0
				Total number of isolates studied =	80

Table 5. Summary of bacterial counts and water temperature of seasonal samples from Interprovincial Pipe Line oil storage tanks

Tank number Trip, Date	Temp (°C)	Total aerobes count/ml	Iron-reducing bacteria count/ml	% total count	MPN/ml	Sulfide-generating bacteria MPN/ml Sulfite-reducers	MPN/ml SRB'S
PEMBINA 23							
2. May-84	10	330	ND *	- **	0.79	150	-
3. Jun-84	13	60	ND	-	35	920	-
5. Aug-84	16	92	TFTC (4) ***	(4%)	-	16 000	490
6. Oct-84	6	60	TFTC (6)	(10%)	-	490	130
7. Jun-85	-	370	20	(5%)	11 000	24 000	-
FEDERATED 19							
2. May-84	10	110	TFTC (1)	(1%)	2.4	350	-
3. Jun-84	14	910	270	(30%)	>160	920	-
4. Jul-84	19	2 450	ND	-	240	4 900	-
6. Oct-84	6	28	ND	-	-	130	230
7. Jun-85	13	2 660	880	(33%)	790	2 800	-

Table 5 (continued)

Tank number Trip, Date	Temp (°C)	Total aerobes count/ml	Iron-reducing bacteria count/ml	% total count	MPN/ml	Sulfide-generating bacteria MPN/ml Sulfite-reducers	MPN/ml SRB'S
COLD LAKE 18							
4. Jul-84	20	1 280	182	(14%)	1 500	220	-
5. Aug-84	18.5	1 100	84	(8%)	-	-	2 200
6. Oct-84	1	152	36	(23%)	-	3 300	330
7. Jun-85	11	610	240	(39%)	490	2 300	-
REDWATER 24							
2. May-84	10	64	ND	-	1.1	2.3	-
3. Jun-84	15	74	TFTC (4)	(5%)	4.9	49	-
WIZARD LAKE 5							
2. May-84	10	4	ND	-	< 0.02	2.3	-
RAINBOW LAKE 2							
2. May-84	10	27	ND	-	<0.02	1.1	-

* ND= none detected
** - = not determined
*** TFTC= present but too few to count (approximate number)

the summer period. Some fields, Redwater, Wizard Lake and Rainbow Lake, had low bacterial counts whereas others, such as Federated and Cold Lake, had consistently higher counts.

All samples contained aerobic bacteria, from a low of 4 to a high of 2.66×10^3/ml. All samples tested contained sulfide-generating or sulfate-reducing bacteria numbering from 1.1 to 2.4×10^4 per ml of fluid examined. The counts of this physiological group of bacteria were usually higher than the counts of iron-reducing bacteria. For samples for which numbers of both sulfide-generators and SRB were determined, the counts of sulfide-generating bacteria were statistically significantly higher ($P<0.05$) than those for SRB in two cases. This may have been either due to the reduction of the medium by the addition of sulfite which helped the growth of SRB, or detection of a large number of sulfite-reducers that were present. The importance of enumerating the sulfite-reducing bacteria in addition to SRB to estimate the sulfide-generating capacity of the population was emphasized by Westlake and Cook (1978) and Obuekwe et al. (1983).

In the samples examined in this study, the distribution of iron-reducing bacteria also varied in a random manner. Iron-reducing bacteria were detected in the MPN tubes for 15 of 18 IPL draw-off water samples and concave, orange colonies were found on B10 agar plates from 11 samples. Counts vary widely with the source of sample, with values ranging from only 1 or 2 colonies (too few to count) up to 8.8×10^2/ml (33% of the total counts of aerobic bacteria). Obuekwe's original iron-reducing isolates came from a survey of the Pembina oil field but in this study, Pembina samples had very low counts of iron-reducers and yielded only 8 isolates. Their presence in Federated samples varied widely from nondetectable to readily detectable numbers in June samples. Redwater samples had low counts but some iron-reducers were found. Highly saline samples, such as those from Wizard Lake (120 ppt) and Rainbow Lake (90 ppt), had low bacterial counts and no iron-reducers were detected. All of the Cold Lake draw-off water samples,

which come from a field which is using steam injection to recover heavy oil, contained significant numbers of iron-reducing bacteria. These organisms were a major component (8-39%) of the total aerobic population.

The numbers of bacteria detected in Esso Norbuck samples are shown in Table 6. Some of these samples had high numbers of aerobic bacteria, and 3 produced waters from around Breton, Alberta had iron-reducers present.

Oil samples collected from pipe lines at the IPL tank farm all had very low total bacterial counts (Table 7). Very low counts of sulfide-generators were found in 3 of 6 Pembina and Federated oil samples. Iron-reducing bacteria were only found in the Cold Lake oil using the B10 MPN soft agar technique although no aerobic bacteria could be isolated from these tubes. B10 soft agar was used for detecting these bacteria in oil samples as the oil was dispersed, resulting in an increase in exposed surface area and thus improving bacterial detection.

Obuekwe (Ph.D. thesis) consistently found iron-reducing and sulfide-generating bacteria in Pembina oil samples. The relatively low incidence of microorganisms in Pembina oil now indicates that procedures, such as control on the bottom sediment and water content (BSW) of oils and the use of biocides, have been implemented which apparently have reduced the bacterial load.

Isolation of iron-reducing bacteria from oil field fluids

Bacteria with the ability to reduce ferric iron have been associated with corrosion of metal in oil pipelines (C.O. Obuekwe, Ph.D. thesis; Westlake et al. 1986). A total of 80 bacterial cultures, which had characteristics similar to Obuekwe's isolate Ps 200, were isolated and purified for further study and all were subsequently identified as being strains of *A. putrefaciens* (see Section B). An additional 45 aerobic bacterial isolates found in oil and produced water which would not reduce ferric iron represented members of the

Table 6. Summary of bacterial counts, salinity and pH of produced water samples from the Esso Norbuck oil field, Drayton Valley, Alberta

Sample no. Well location	Total aerobes counts/ml	Iron-reducing bacteria counts/ml	(% total count)	SRB MPN/ml	pH	Salinity (ppt)
BRETON Nov-84						
1. 4-2-48-4W5	32 000	2 300	(1%)	1.3	7.2	13
2. 10-35-47-4W5	4 230 000	ND*	- **	17	7.4	9.5
3. 12-26-47-4W5	470	TFTC (1)***	(<1%)	170	7.2	8
4. 16-23-47-4W5	36 000	5 000	(1%)	3.3	7.1	11
9. 19-35-47-4W5	1 190	ND	-	-	7.1	11
LINDALE Nov-84						
5. 14-18-49-5W5	520	ND	-	<0.2	-	-
6. 14-27-49-6W5	126 000	ND	-	23	-	-
7. 16-33-49-6W5	310 000	ND	-	3.3	-	-
8. 16-19-49-5W5	76 400	ND	-	0.45	-	-
10. 8-13-49-6W5	3 400 000	ND	-	-	-	-

* ND= none detected

** - = not determined

*** TFTC= present but too few to count (approximate number)

Table 7. Summary of bacterial counts and temperature of oil from pipelines flowing into the IPL Edmonton terminal

Pipeline sampled Date	Temp. (°C)	Total aerobes count/ml	Iron-reducing bacteria count/ml	Iron-reducing bacteria MPN/ml	Sulfide-generating bacteria MPN/ml Sulfite-reducers	Sulfide-generating bacteria MPN/ml SRB'S
PEMBINA						
Feb-84	8	TFTC*	ND **	ND	2.2	- ***
Aug-84	17	24	ND	-	3.3	ND
Mar-86	-	ND	-	ND	ND	-
FEDERATED						
Feb-84	0	TFTC	ND	ND	ND	-
Aug-84	13	ND	ND	-	0.2	ND
Mar-86	-	ND	-	ND	ND	-
COLD LAKE						
Jun-85	9	ND	ND	0.78	ND	-
Mar-86	-	ND	-	2.3	ND	-
WIZARD LAKE						
Feb-84	9.5	TFTC	ND	ND	ND	-
ELLERSLIE						
Mar-86	-	ND	-	ND	ND	-

* TFTC= present but too few to count (approximate number)
** ND= not detected
*** - = not determined

genera *Pseudomonas, Alcaligenes, Bacillus, Flavobacterium, Micrococcus* and *Corynebacterium.*

The majority of iron-reducing cultures were isolated on B10 plates which yielded concave, orange colonies varying in size and shape. Some were small (4-5 mm), smooth, opaque colonies with varying diameter of concave centres while others were larger (10mm), spreading, transluscent types, some with inclusions and some with dark, green centres. Potential iron-reducing isolates from PCA plates were convex in shape, ranged in color from salmon pink to dark orange, and some had smooth edges while others were large, spreading colonies. Records were kept of the source and approximate number of each type of colony and all were confirmed as being iron-reducers, in B10 broth.

Initially, three cultures (T2Pem 12, T2Fed 3 and T2Fed 4) were isolated aerobically from positive B10 MPN tubes because very few colonies grew on the plates from Trip 2 samples (Table 5). This method was abandoned because it was difficult to isolate and purify individual colonies. Anaerobic incubation of B10 agar plates streaked from cultures in MPN tubes yielded numerous facultative and anaerobic organisms with colonial morphologies varying in color and shape from creamy white and spreading to shiny, black punctiform colonies. Facultative iron-reducing bacteria, such as isolate Ps 200, grew anaerobically on B10 agar plates and formed small, white colonies which developed green crust upon exposure to air. These isolates would not grow anaerobically on PCA or O/F plates, because of a lack of a suitable electron acceptor. Strictly aerobic *Pseudomonas aeruginosa* ATCC 10145, which did not reduce ferric iron, would not grow anaerobically on either B10 medium or PCA. Four isolates, designated AN22, AN24, AN25 and AN29, which formed concave colonies when grown aerobically on B10, were isolated anaerobically from samples taken from Pembina, Federated and Redwater on Trip 3, June 1984. Additional iron-reducing organisms, isolated

anaerobically from oil field environments, included 3 lactose-fermenting *Enterobacter* sp., 1 coryneform and 5 *Clostridium* sp. Anaerobic *Clostridium* spp. also were found, as isolated colonies, in soft agar MPN tubes from Cold Lake oil (Table 7) and Cold Lake, Pembina and Federated waters taken on Trip 7, June 1985 (Table 5).

Comparison of iron-reducing isolates

The 80 culture collection strains reduced a wide range of terminal electron acceptors including ferric iron, nitrate and thiosulfate, and 90% of the isolates reduced sulfite. The terminal electron acceptors used by these *A. putrefaciens* strains, the other iron-reducing isolates and strains of *Pseudomonas* sp. from oil fields, as well as *P. aeruginosa* ATCC 10145 and *Proteus vulgaris* ATCC 13315 are compared in Table 8.

A correlation between nitrate reduction and iron reduction was suggested by Ottow (1969a, 1970; Ottow and Glathe 1971). However, coryneform AN13 reduces ferric iron but has no nitrate reductase activity and five *Pseudomonas* sp. isolated from produced water and *P. aeruginosa* ATCC 10145 have nitrate reductase activity but do not reduce ferric iron. Other researchers such as Jones et al. (1984a) and Pfanneberg and Fischer (1984) also have isolated iron-reducers which did not reduce nitrate and vice versa. Sulfide-generating, nitrate-reducing *P. vulgaris* ATCC 13315 did not reduce iron either. The only other bacteria found in this study with the combined ability to reduce sulfite and thiosulfate as well as ferric iron were the anaerobic clostridia.

Isolates identified as *Clostridium* spp. would not grow aerobically, produced spores and did not reduce sulfate when grown in modified Butlin's medium. Iron-reducing clostridia are commonly isolated from soil and sediments and the characteristics of strains of *Clostridium* sp. have been studied by Munch and Ottow (1982, 1983; Ottow and Munch 1978) and Hamman and Ottow (1974). Sulfite reduction also appears to be widespread amongst the clostridia (Laishley et al. 1984). Since they

Table 8. Alternate electron acceptors used by oil field isolates and known bacterial strains

	Electron acceptor reduced				
	Fe(III)	$SO_3^=$	$S_2O_3^=$	Nitrate to	
Oil field isolates (# strains)	to Fe(II)	to $S^=$		to NO_2^-	to Gas
Alteromonas putrefaciens (80)	+	+ (90%)	+	+	-
Coryneform (AN 13)	+	-	-	-	-
Enterobacter sp. (AN 27)	+	-	-	+	+
Clostridium sp. (6)	+	+	+	nd[a.]	nd
Pseudomonas sp. (5)	-	-	-	+	-
P. vulgaris ATCC 13315	-	-	+	+	-
P. aeruginosa ATCC 10145	-	-	-	+	+

a. nd= not determined

are readily isolated from oil and produced water, they could form a part of the bacterial population that contributes to corrosion along with the *A. putrefaciens* strains.

The lactose-fermenting strains isolated from water samples were identified as *Enterobacter* sp. but as they do not form concave, orange colonies when grown aerobically on B10 plates, they would not have been detected by the aerobic spread-plate technique used in this study. Lactose-fermenting, iron-reducing, gram-negative rods are commonly isolated from soils (Bromfield 1954; Ottow 1969b; Ottow and Glathe 1971) and have also been isolated from the oil field environment (Dr. F.D. Cook, University of Alberta, personal communication). Iron reduction by various members of the Enterobacteriaceae has been studied by Ottow (1969a, 1968) and Lundgren et al. (1983).

A coryneform isolate, AN13, formed punctiform, yellow colonies with depressed centres on B10 medium and would also have been overlooked on aerobic plates. This isolate was a gram-variable, non-motile, slightly pleomorphic coccobacillus. It produced acid from glucose fermentatively, was oxidase negative and catalase positive. Ferric iron was rapidly reduced in B10 medium, but it did not reduce nitrate, sulfite or thiosufate. Iron-reducing coryneforms have been isolated (Ottow and Glathe 1971) and an isolate designated *Corynebacterium ferrireductans* has been studied by Pfanneberg and Fischer (1984).

Members of the genus *Bacillus* are common iron-reducing isolates present in soil (Ottow 1969a; Ottow and Glathe 1971) and have been previously isolated from oil field samples (F.D. Cook, personal communication). Some strains have been reported to form concave colonies (C. Panter, M.Sc. thesis), but no iron-reducing *Bacillus* spp. were found during this study.

The ability to reduce ferric iron has been reported in a number of other *Pseudomonas* spp. (Ottow 1969a; Ottow and Glathe 1971), however no pseudomonads capable of reducing ferric iron were isolated from oil fields surveyed in this study.

Iron-reducing organisms with biochemical characteristics similar to those of isolate Ps 200 have not been described in the literature. While other types of iron-reducing bacteria are found in the oil field, this study concentrates on the group of orange-pigmented organisms similar to Obuekwe's corrosion-causing isolate Ps 200 which represent a substantial portion of the total iron-reducing population.

B. CHARACTERIZATION AND IDENTIFICATION OF IRON-REDUCING BACTERIA

Characteristics of oil field isolates

A summary of the biochemical characteristics used in classifying oil field isolates as strains of *A. putrefaciens* are presented in Table 9. A list of individual culture collection isolates, ATCC strains and their characteristics is given in Appendix E. All oil field isolates were gram-negative rods with a single polar flagellum, oxidase and catalase positive and produced a salmon pink to orange pigment. All of the isolates examined, including those from the ATCC, reduced ferric iron. All produced sulfide from thiosulfite in defined medium or when grown on TSI medium (Difco) and 90% reduced sulfite. Of the 59 isolates tested, all reduced nitrate to nitrite. Screening of these isolates with the API 20E identification system tentatively identified them as being strains of *P. putrefaciens*. All of the isolates subsequently tested positive for extracellular DNase and L-ornithine decarboxylase. The mol% G+C values for selected isolates were determined (Appendix B) and ranged from 42.4-56.0%, within the range of values reported for the genus *Alteromonas* (Baumann et al. 19[illegible] Lee et al. 1977).

All of the isolates produced a weak acidic or an alkaline reaction when grown on glucose and an alkaline reaction on lactose agars, reactions which are typical of *Alteromonas* spp. (Lee et al. 1977; Hendrie and Shewan 1979). Trimethylamine oxide was reduced to trimethylamine, which is a diagnostic test for *A. putrefaciens* (Lee et al.

Table 9. Characteristics of *Alteromonas putrefaciens* strains isolated from oil field samples

Characteristic	Result (# positive / # tested)[a.]
Pigment	salmon pink to orange
Flagella	polar, monotrichous
Oxidase	+
Catalase	+
O/F glucose	oxidative, no acid (alkali) (74 / 80)
Electron acceptors reduced	
Ferric iron reduction	+
H_2S production from $S_2O_3^=$	+
$SO_3^=$	+ (71 / 80)
Nitrate reduction to nitrite	+ (59 / 59)
Trimethylamine oxide reduced	+ (31 / 31)
Extracellular hydrolases	
DNase	+ (31 / 31)
Gelatin hydrolysis	+ (32 / 38)
Starch hydrolysis	- (0 / 30)
Amino acid decarboxylases	
Ornithine decarboxylase	+ (31 / 31)
Arginine dihydrolase	- (0 / 26)
Lysine decarboxylase	- (0 / 26)
Variable results	
Citrate utilization	± (16 / 43)
Urease production	± (10 / 29)
Growth at 4°C	± (9 / 43)
Growth on 4.5% NaCl	+ (43 / 43)
6% NaCl	± (35 / 43)
7.5% NaCl	± (33 / 43)
mol% G+C	42.4 - 56.0 (16)

a. otherwise all (80 / 80) were positive

1977). This compound serves as an alternate electron acceptor for the anaerobic growth of this organism during fish spoilage (Ringo et al. 1984). All cultures were positive for gelatin hydrolysis but were unable to use starch and did not show lysine decarboxylase or arginine dihydrolase activity. Varied responses were obtained with regards to the utilization of citrate and urease as well as salt tolerance and growth at 4°C.

Based on all of these reactions, these isolates could be placed in the genus *Alteromonas*, and are clearly identifiable as strains of *A. putrefaciens* (Hendrie and Shewan 1977; Hugh and Gilardi 1980; Semple and Westlake 1987a). These oil field isolates are similar to those in the sub-cluster Phenon E of Lee et al. (1977) which have a mol% G+C content in the range 43-55%, produce H_2S, DNase and ornithine decarboxylase, reduce trimethylamine oxide and do not have a requirement for sodium ions for growth.

Fifteen isolates were tested for growth on crude oil as the sole carbon source. They emusified the oil and the medium became turbid. However, analysis of the oil using gas chromatography techniques showed no changes in the sulfur heterocycle or saturate fractions. There was a slight reduction in peak height of only 2 or 3 peaks in the aromatic profile, in the areas where biphenyl and methylbiphenyl are resolved. Other isolates with this capability, identified as *Alcaligenes* sp. and *Acinetobacter* sp., have been described by Fedorak and Westlake (1983).

Differentiation of oil field strains

The characteristics used to differentiate individual *A. putrefaciens* isolates are shown in Table 10. The oil field isolates and ATCC type strains studied can be divided as described by Owen et al. (1978) into four groups based on their abilities to grow at 4°C and at various salt concentrations. They can be further differentiated because groups 1

Table 10. Differential characteristics of oil field and ATCC strains of *A. putrefaciens*

Isolate	Growth at 4°C	Growth + NaCl (25°C) 4.5%	Growth + NaCl (25°C) 6.0%	Growth + NaCl (25°C) 7.5%	Hydrogen sulfide production from thiosulfate	Hydrogen sulfide production from sulfite	Acid glucose	Simmons citrate	Urease	mol% G+C
GROUP 1										
ATCC 8071	+	+	-	-	+	+	-	-	-	43.8*
ATCC 12099	+	+	-	-	+	+	-	-	-	43.6*
ATCC 19857	+	+	-	-	+	+	-	-	-	44.2*
Ps 200 (Pem)	+	+	-	-	+	+	-	-	-	42.4
216 (Pem)	+	+	-	-	+	+	-	-	-	45.4
230 (Pem)	+	+	-	-	+	+	-	-	-	nd**
T2Pem 12	+	+	-	-	+	+	-	-	-	42.8
									Avg. =	43.7±1.6
GROUP 2										
ATCC 8072	+	+	-	-	+	-	+	+	-	47.2*
213 (Pem)	+	+	-	-	+	-	+	-	-	44.2
CL70	+	+	-	-	+	-	+	-	-	48.6
ESSO 4-1	+	+	-	-	+	-	+	+	-	43.8
ESSO 4-2	+	+	+	-	+	-	+	+	-	nd
ESSO 1-1	+	+	-	-	+	-	-	+	-	44.8
									Avg. =	45.7±2.4
GROUP 3										
ATCC 8073	-	+	-	-	+	-	+	-	-	46.1*
ESSO 1-3	-	+	+	-	+	-	-	+	-	50.8
									Avg. =	48.5±2.4

Table 10. (continued)

Isolate	Growth at 4°C	Growth + NaCl (25°C) 4.5%	6.0%	7.5%	Hydrogen sulfide production from thiosulfate	sulfite	Acid glucose	Simmons citrate	Urease	mol% G+C
GROUP 4										
T2Fed 2	-	+	+	+	+	+	-	+	+	nd
T2Fed 3	-	+	+	+	+	+	-	+	+	49.4
T2Fed 4	-	+	+	+	+	+	-	+	-	nd
T3Fed 4	-	+	+	+	+	+	-	+	+	nd
AN22 (Fed)	-	+	+	+	+	+	-	-	-	55.2
AN29 (Fed)	-	+	+	+	+	+	-	-	-	nd
AN24 (Red)	-	+	+	+	+	+	-	+	+	51.2
CL31	-	+	+	+	+	+	-	-	-	54.2
CL33	-	+	+	+	+	+	-	+	-	nd
CL36	-	+	+	+	+	+	-	-	-	nd
CL41	-	+	+	+	+	+	-	+	-	51.4
CL 71	-	+	+	+	+	+	-	+	+	56.0
Fed 75	-	+	+	+	+	+	-	+	-	55.6
Fed 80	-	+	+	+	+	+	-	-	+	51.8
ESSO 1-7	-	+	+	+	+	+	-	-	+	nd
ESSO 1-8	-	+	+	+	+	+	-	-	+	nd
									Avg. =	53.1±2.4

* mol% G+C value reported by Owen et al. 1978

** nd= not determined

and 4 isolates reduce sulfite and those in groups 2 and 3 do not. All isolates, however, readily produced sulfide from thiosulfate and it is probable that in group 2 and 3 isolates, the sulfide originated from the sulfane sulfur (Chambers and Trudinger 1979) of thiosulfate as these cultures cannot reduce sulfite (Obuekwe et al. 1983). Ninety percent of all the strains isolated reduced sulfite, and based on detailed count data, isolates showing group 4 characteristics predominated in the produced fluid samples examined. The cultures studied in this survey showed a varied response with regards to their ability to produce acid from glucose, grow on citrate and produce urease. The mol% G+C composition of isolates increased from an average of 43% in group 1 to 53% in group 4. The average mol% G+C calculated for each group is similar to those reported by Owen et al. (1978) who used a similar scheme to classify isolates of *A. putrefaciens* into DNA homology groups. A summary of the characteristics of these groups is presented in Table 11.

Several studies (Holmes et al. 1975; Levin 1972; and Riley et al. 1972) have reported that *A. putrefaciens* isolates can be divided into a salt tolerant group that will grow in the presence of NaCl concentrations greater than 6% and others that will not. As the isolates investigated in this study were obtained from oil field fluids with salinities ranging from 8 ppt to 71 ppt, a survey of their salt tolerance was included in this study. All isolates grew without the addition of NaCl to the media and would also grow in the presence of 4.5% NaCl (i.e. they were halotolerant). Only 2 isolates, ESSO 4-2 (group 2) and ESSO 1-3 (group 3), of the first three groups grew in the presence of 6% NaCl whereas all of the isolates in group 4 grew in the presence of both 6% and 7.5% NaCl. An additional 17 group 4 isolates were tested and grew well on 6% NaCl and to varying degrees on 7.5% NaCl. That is, group 4 isolates, the predominant group, could not grow at 4°C but are relatively halotolerant whereas those isolates in groups 1 and 2 are only mildly halotolerant but could grow at 4°C. Only isolate ESSO 1-3 and *A. putrefaciens* ATCC 8073, were mildly halotolerant but did not grow at 4°C; that is, they were

Table 11. Summary of characteristics of groups of *A. putrefaciens*

Group	Growth 4°C	Growth + NaCl 4.5%	Growth + NaCl 7.5%	H_2S production from $S_2O_3^=$	H_2S production from $SO_3^=$	Glucose acid	Simmons citrate	Urease	mol% G+C
1	+	+	-	+	+	-	-	-	43.7±1.6
2	+	+	-	+	-	+	± [a.]	-	45.7±2.2
3	-	+	-	+	-	±	±	-	48.5±2.4
4	-	+	+	+	+	-	±	±	53.1±2.4

[a.] ± = variable response

intermediate in character between groups 1, 2 and 4. Based on these results and those in the literature (Lee et al. 1977; Riley et al. 1972), the use of degrees of halotolerance as a taxonomic criterion for subdividing strains of *A. putrefaciens* is of questionable value. Lee et al. (1977) studied the salt tolerance of two subclusters of Phenon E (*A. putrefaciens* strains) but could not divide their isolates based on their ability to grow in 6% NaCl. Riley et al. (1972) also reported a wide range of salt tolerance in clinical isolates of *A. putrefaciens*. Cultures exhibiting varying degrees of halotolerance have also been isolated from oil field samples.

Distribution of physiological groups of *Alteromonas putrefaciens*

Information in the literature on the distribution of *A. putrefaciens* has suggested that groups 1 and 2 isolates, which grow at low temperatures, tend to be isolated from spoiling fish and meat (Parker and Levin 1983; McMeekin and Patterson 1975). The more salt tolerant group 3 and 4 isolates, which grow at higher temperatures, are predominantly from human clinical specimens (Levin 1972; Holmes et al. 1975; Owen et al. 1978). In the present study, group 4 isolates were most common and were obtained from all the oil field fluids examined. Detailed count data show that all isolates from Federated samples and more than 95% of the Cold Lake isolates were salt tolerant (group 4) whereas groups 1 and 2 type isolates were found only in lower salinity samples (Pem, CL, Esso).

Comparison to salt-requiring *Alteromonas* sp.

Several cultures which require a seawater base for growth and which are similar to the *Alteromonas* subcluster C of Lee et al. (1977) were tested for iron reduction and sulfide production from sulfite and thiosulfite. These included pigmented *Alteromonas aurantia* ATCC 33046, *Alteromonas rubra* ATCC 29570, *Pseudomonas piscicida* ATCC

15251 and non-pigmented *Alteromonas haloplanktis* ATCC 14393, *Alteromonas macleodii* ATCC 27126 and *Pseudomonas atlantica* ATCC 19262. None of these isolates carried out either reaction, indicating that physiologically this subcluster is quite different from subcluster E which contains *A. putrefaciens* strains. These cultures, except *A. aurantia*, also tested DNase positive, which differentiates the genus *Alteromonas* from *Pseudomonas* (Hendrie and Shewan 1979), but were ornithine decarboxylase negative and did not reduce TMAO (Appendix E).

Studies by Van Landshoot and DeLey (1983) differentiated the various *Alteromonas* sp. of the basis of rRNA/DNA hybridization and concluded that *A. putrefaciens* was sufficiently different from other *Alteromonas* sp. to represent a separate branch of the genus. The four separate DNA homology groups also were distinguished by this method. Included in the branch were a luminescent bacterium *Alteromonas hanedai* (Jensen et al. 1980) and agar-degrading *Pseudomonas atlantica*. Phylogenetic studies based on analysis of 5s and 16s rRNA sequences places *A. putrefaciens* in the gamma 3 subdivision of the purple photosynthetic bacteria and their relatives (Woese et al. 1985), closely related to the vibrios and enteric bacteria. Also in this group are a number of sulfur-oxidizing bacteria and iron-oxidizing thiobacilli (Stahl et al. 1987). Woese (1985) speculated on the close evolutionary relationships between bacteria with various oxidizing and reducing metabolisms, implying that the two metabolic processes are closely related and that evolution turned them in either the oxidative or reductive direction. An interesting phylogenetic relationship was found between *A. putrefaciens* and two deep sea, barophilic strains designated *'Vibrio benthica* ', based on their sequences of the 5s rRNA they contain (Deming et al. 1984; Stahl et al. 1987). MacDonnell and Colwell (1985) have gone so far as to propose a new genus, *Shewanella*, for *A. putrefaciens* and these other organisms which share an evolutionary history and which are distinct from other *Alteromonas* spp. (Van Landshoot and DeLey 1983). Analysis of the 5s rRNA sequences has only been reported for one group 1

strain, ATCC 8071, and analysis of strains from other groups should be performed to confirm this hypothesis. The other organisms to be included in this genus, salt-requiring *A. hanedai*, and the fermentative *V. benthica*, should also be tested for iron-, sulfite- or TMAO-reducing ability. Information gathered on the 80 oil field strains should be useful for classification of the genus.

Summary

Strains of *A. putrefaciens* have been readily isolated from the oil field fluids examined in this study. These isolates exhibited varying degrees of halotolerance, were capable of reducing iron and producing sulfide and some were able to grow at 4°C. The variation in biochemical characteristics exhibited by these organisms, such as a range of growth temperatures, degrees of halotolerance and their unique reductive capabilities, indicate that they could contribute to corrosion in diverse oil field environments. The fact that all isolates tested were capable of ferric iron reduction and sulfide generation, properties not found in the salt-requiring alteromonads or in all pseudomonads, suggests that *A. putrefaciens* strains are a readily identifiable intermediate group, readily distinguishable from other gram-negative, aerobic rods.

C. EVALUATION OF FERRIC IRON REDUCTION ACTIVITY

Development of a soluble ferric iron reduction assay

Iron-reducing isolates were initially screened for iron-reducing activity of growing cells in B10 medium using the comparative procedure of Obuekwe (Ph.D. thesis), which measures the total iron produced over a 3 hour incubation period. Initial studies indicated that in order to compensate for slight differences in lag time before iron reduction begins and the early leveling off of ferrous ion production at high reduction rates, the rate of ferrous ion production should be measured. Further studies using a defined assay

system, with lactate as the carbon source and resting cells rather than growing cells (Obuekwe et al. 1982b), showed that because of differences in growth characteristics of different isolates, iron reduction rates could not be compared on the basis of cell number, optical density or dry weight. However, useful data could be obtained by comparing iron-reducing activity based on the protein concentration of resting cells. Data for iron reduction experiments with 19 isolates are given in Appendix F.

Growth curves of 4 iron-reducing strains, grown aerobically at 27°C in Butlin's medium, are shown in Figure 8. These isolates showed similar growth patterns with slight differences in lag time, growth rate and the final optical density reached. The changes in OD per hour during linear growth (i.e. between about 0.2 and 1 OD units), were similar for all isolates (Ps 200 = 0.263, Pem 12 = 0.237, T2Fed 3 = 0.248, CL41 = 0.229). The average for 7 tests on 5 isolates was $\Delta OD/h = 0.245 \pm 0.020$.

Resting cell suspensions were routinely prepared from cultures grown for 13 to 15 hours, which were in late exponential phase to early stationary phase of growth. The 19 isolates tested had a final OD's ranging from 0.59 to 1.57 (Appendix F). Cell suspensions were prepared using 1 g wet weight of cells in 80 ml buffer which gave protein concentrations ranging from 255 to 741 µg protein/ml. Cell numbers varied widely from 3.3×10^7 up to 7×10^9 /ml. The use of increasing volumes of resting cell suspension (from 0.5 to 3 ml), yielded utilizable iron reduction rates for all strains tested. The data in Figure 9a shows the ferrous iron production with time for varying concentrations of resting cells of isolate Ps 200. In preliminary experiments, assays were carried out in triplicate and the standard deviation of the iron concentration measured at each time point ranged from 2 to 10 mg Fe^{2+}/l. All further experiments were performed using one tube for each concentration of cells being tested. Rates of ferrous ion production were calculated from the slope of the curve using a linear regression program, to ensure that correlation coefficients were always greater than r=0.99. The data in Figure

Figure 8. Aerobic growth of four iron-reducing isolates of *A. putrefaciens* at 27°C in modified Butlin's medium.

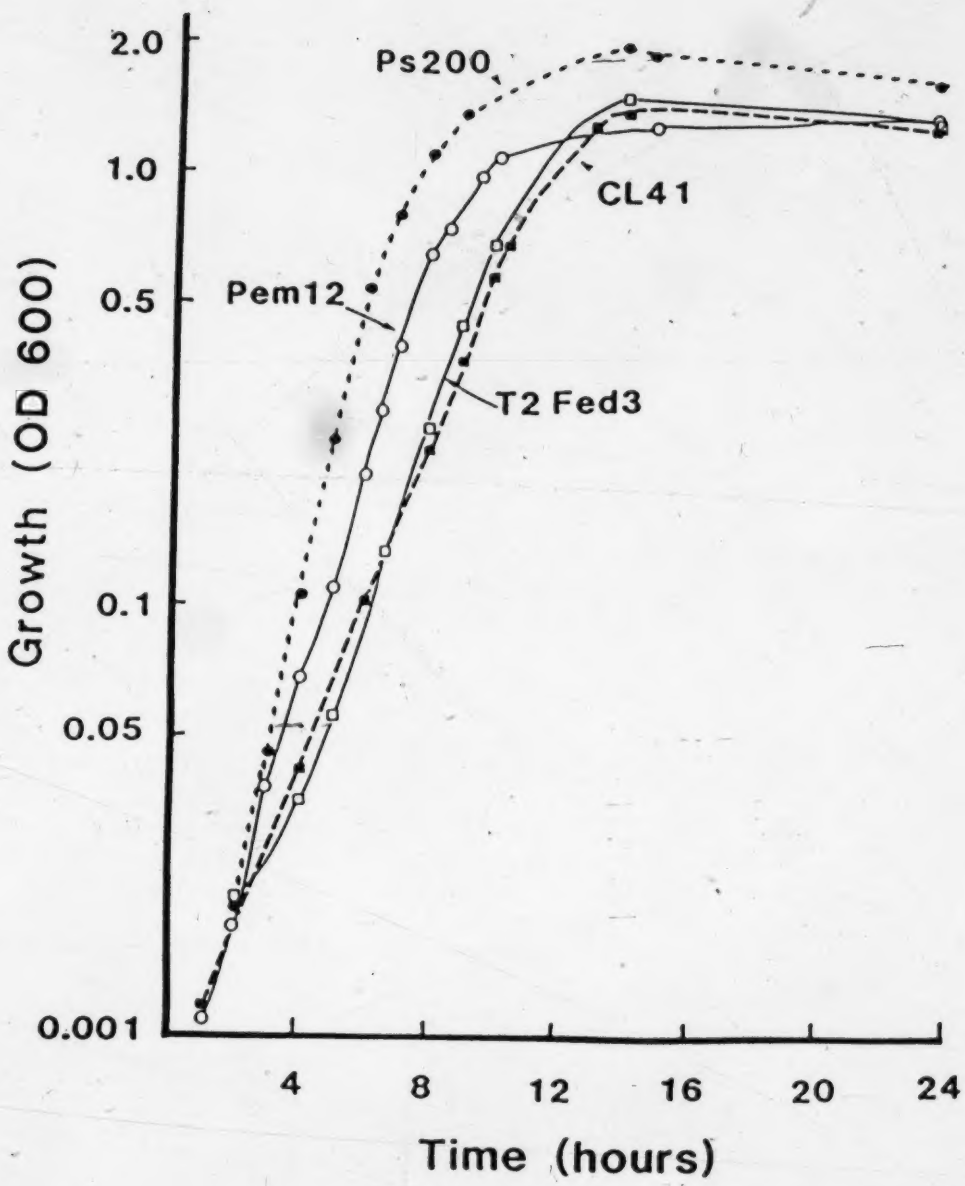
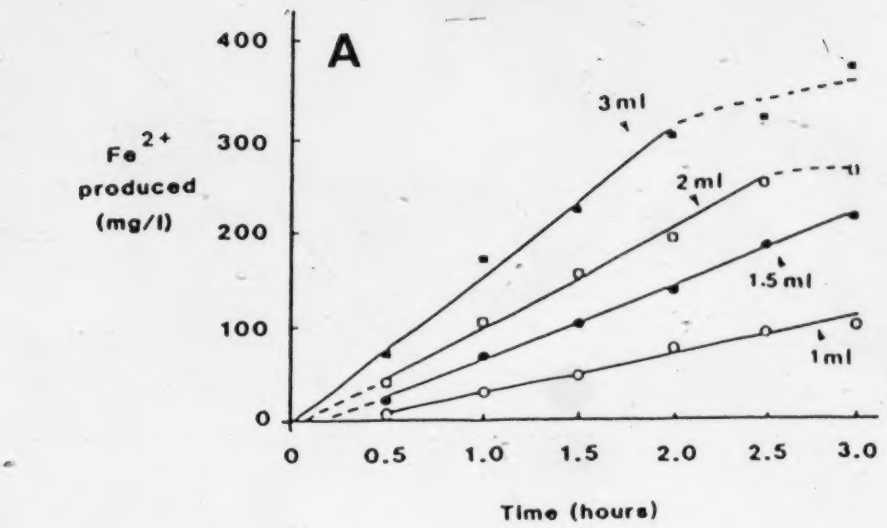
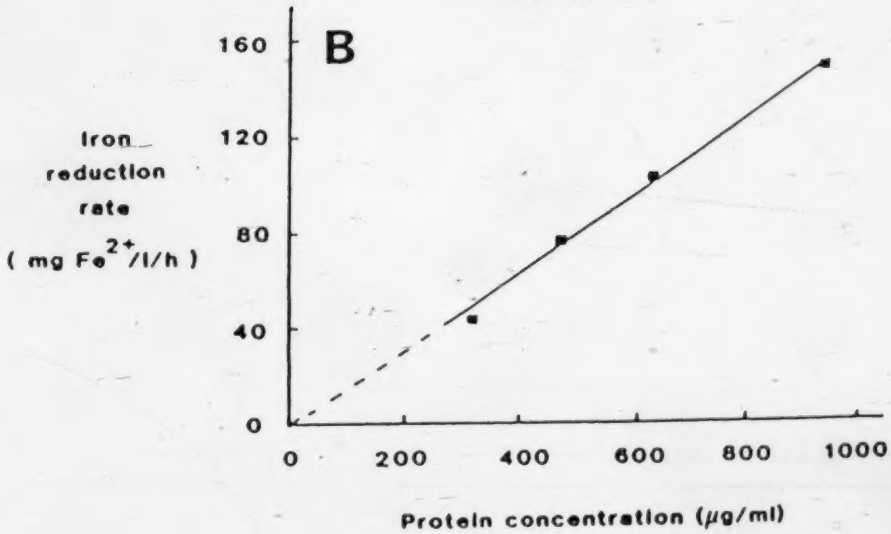

Ps200
CL41
Pem12
T2 Fed3
Growth (OD 600)
2.0
1.0
0.5
0.1
0.05
0.001
4
8
12
16
20
24
Time (hours)

Figure 9. Iron reduction by increasing volumes of resting cells of *A. putrefaciens* isolate Ps 200.

A. Note that ferrous ion production levels off between 300 and 400 mg Fe^{2+}/l. Rates greater than about 130 mg Fe^{2+}/l /h would require more time points for accurate measurement.

B. Increase in iron reduction activity was proportional to increase in protein concentration for all isolates tested up to 1500 µg/l protein. Iron reduction per unit protein was calculated for rates between 20 and 130 mg Fe^{2+}/l/h.


A
400
300
200
100
0
Fe^{2+} produced (mg/l)
3 ml
2 ml
1.5 ml
1 ml
0
0.5
1.0
1.5
2.0
2.5
3.0
Time (hours)


B
160
120
80
40
0
Iron reduction rate (mg Fe^{2+}/l/h)
0
200
400
600
800
1000
Protein concentration (µg/ml)

9b shows a linear increase in iron reduction rate with increased protein concentration. A linear increase in iron reduction rate was found up to approximately 1500 µg protein/ml for all of the isolates tested. Reduction rates between about 20 and 130 mg Fe^{2+}/l/h, which showed a constant rate of ferrous iron production for 3 hours, were used for calculation of iron-reducing activity/mg protein. Higher rates (160-200 mg Fe^{2+}/l/h) were estimated to determine the activity vs. protein content curve for each isolate; shorter sampling times would have to be used for more accurate rate determinations. At ferrous ion concentrations between about 300 and 400 mg Fe^{2+}/l, the rate of Fe^{2+} release levelled off before accurate rates could be determined and the medium changed from green to white, with some precipitation occurring. Interference with iron reduction assays by phosphate, which precipitates the ferrous iron, has been reported by Arnold et al. (1986a, 1986b). Iron phosphate precipitates [$Fe_3(PO_4)_2 \cdot 8H_2O$, vivianite] in B10 medium were noted by Panter (M.Sc. thesis) and by Pfanneburg and Fischer (1984) during the reduction of the insoluble ferric oxides lepidocrocite and goethite.

The effect of the growth phase on the rate of iron reduction of resting cells was tested with isolates Ps 200 and T2Fed 3 (Table 12). Separate batches of cells were harvested at different times during different growth phases. Although the resting cell preparations varied widely in cell number and protein concentration, iron reduction rates per unit protein for exponential and stationary phase cells were approximately the same for the three batches of isolate Ps 200 tested. For T2Fed 3, exponential and early stationary phase cells showed higher reduction rates than stationary phase cells. Since there were no differences in the calculated rates between exponential and early stationary phase cells for either isolate, further assays were done with cells harvested while the OD was still increasing as previously discussed.

Based on a study of 10 isolates, the optimum temperature for iron reduction was 30°C, as reported by Obuekwe (Ph.D. thesis). The assays were carried out at pH 7.2 as

Table 12. Comparison of iron reduction rates of resting cells of exponential and stationary phase cultures of two strains of *A. putrefaciens*

Isolate	Harvest time (OD 600) Growth phase	Viable cell number (/ml)	Protein concentration (µg/ml)	Protein concentration (mg/10^7cells)	Vol. cells (ml)	Iron reduction rate mg Fe^{2+}/l /h	Iron reduction rate /mg protein[a]
Ps 200	6 h (0.88) exponential	1.68×10^8	295 ± 13	17.6	0.5	20	b.
					1	45	153
					2	97	164
					3	159	-
							Avg. = 158
	15 h (1.6) stationary	3.85×10^9	432 ± 30	1.1	0.5	30.8	142
					1	63.6	147
					2	166	-
					3	280	-
							Avg. = 144
	15 h (1.57) stationary	9.70×10^8	624 ± 36	6.4	1	97.4	156
T2Fed 3	14 h (0.92) exponential	6.00×10^7	306 ± 6	51.0	0.5	10.6	-
					1	22.3	73
					2	61	100
					3	103.3	113
							Avg. = 94
	17 h (1.2) stationary	1.80×10^8	469 ± 30	26.1	0.5	0	-
					1	27.2	58
					2	53.7	57
					3	82	58
							Avg. = 58
	15 h (0.93) early stationary	NG[c]	540 ± 20	-	1	51.5	95

a. Iron reduction rates per unit protein were calculated for rates between 20 and 130 mg Fe^{2+}/l /h
b. - = not calculated
c. NG= no growth

described by Obuekwe (Ph.D. thesis). The optimum pH for iron reduction was not determined but it was noted that at low pH (~4.5), iron reduction was severely inhibited, and at high pH, the ferric iron would precipitate from solution.

The effect of lactate concentration on iron reduction was tested with three isolates, including Ps 200. For all isolates, the iron reduction rates increased linearly with increasing lactate concentration, up to 30 µmol/ml, and then leveled off. At higher lactate concentrations, up to 60 µmol/ml, rates continued to increase slowly for isolates T2Fed 3 and Pem 12. For isolate Ps 200, a slight decrease in rate was seen at 60 µmol/ml. Obuekwe (Ph.D. thesis) reported a dramatic inhibition of iron reduction at lactate concentrations higher than 30 µmol /ml with isolate Ps 200. A lactate concentration of 30 µmol/ml, as used by Obuekwe (Ph.D. thesis), gave the most consistent results and was used in all assays.

Obuekwe (Ph.D. thesis) found that the iron reduction rates of resting cells of isolate Ps 200 were constant for a period of 4 weeks if stored in 0.1 M phosphate buffer at 4°C. The effects of similar storage on resting cells of isolates Ps 200, Pem 12 and ESSO 4-1 were investigated to determine the stability of their iron reduction capability. Changes in iron reduction rates, cell number and optical density were tested at various times, from 1 hour to 11 days after the preparation of cell suspensions. Cells remained viable for the storage period, with a only a slight decrease in number and optical density over time (Appendix F). The maximum reduction rates were found with freshly prepared cells and reduction rates tended to decrease with increasing length of storage time. Therefore all assays were carried out within 1-2 hours of resting cells preparation. With longer storage times, an increase in lag times before iron reduction began was also noted.

Isolates listed in Table 13 were tested for changes in iron reduction rate, cell number and cell protein for up to a 3 day storage period. Although iron reduction rates decreased with time, cells remained viable and the protein content remained constant. A

Table 13. Effect of storage at 4°C in 0.1 M phosphate buffer on iron reduction rates of resting cells of *A. putrefaciens* strains

Isolate	Storage time (days)	Viable cell number (/ml)	Total protein content (µg/ml cells)	Iron Reduction Rate @ 30°C (mg Fe^{2+}/l /h /mg protein)[a.]	
Ps 200	0	9.7×10^8	623 ± 36	156	
	2	2.0×10^8	613 ± 8	112	(28%) [b.]
	3	1.9×10^8	649 ± 8	67	(57%)
Pem12	0	2.1×10^9	547 ± 16	234	
	2	7.0×10^8	526 ± 8	145	(38%)
	3	8.8×10^8	534 ± 8	91	(61%)
CL 41	0	3.3×10^7	474 ± 16	95	
	2	2.0×10^7	461 ± 10	14	(84%)
T2Fed 3	0	NG [c.]	540 ± 20	95	
	2	4.0×10^7	542 ± 20	29	(69%)

a. In all cases, the reaction volume was 10 ml so that iron reduction activity expressed as µmol Fe^{2+}/h/mg protein can be calculated by dividing by a factor of 5.58
b. % decrease in reduction rate compared to freshly prepared cells (Day 0)
c. NG= no growth

total of 11 isolates tested varied greatly in their sensitivity to storage, with reduction rates decreasing from only 3% up to 84% after 2 days storage (Appendix F). Isolates CL41 and T2Fed 3 were more sensitive to storage than other isolates tested (Table 13). Iron reduction rates decreased so much with these isolates that no iron reduction was measured with 1 ml of cells and rates could only be determined by using larger volumes of resting cells. These results emphasize the importance of testing a range of protein concentrations when determining rates of iron reduction.

Comparison of iron reduction rates of *A. putrefaciens* isolates

The reduction rates determined for late exponential phase cells of 19 isolates of *A. putrefaciens* are shown in Table 14. The cultures reduced soluble ferric ions at rates varying from 42 up to 237 mg Fe^{2+}/l/hr/mg protein (7.5 to 42.5 µmol Fe^{2+}/h/mg protein). The reduction rate data for different volumes of cells, as well as protein content and cell number for all isolates tested are given in the Appendix F. There were no striking differences in growth characteristics, final cell number or protein concentrations between the groups of isolates.

There were no significant differences in the iron reduction rates of the different groups of *A. putrefaciens* isolates. In B10 broth tubes, the color changes from gold to green were sometimes more rapid and intense for Group 1 and 2 isolates than for Group 4 isolates, but the former did not appear to have a significantly higher rate of iron reduction in this assay. Although Group 4 isolates T2Fed 3 and CL41 had somewhat lower reduction rates than isolate Ps 200 (as shown in the experiments listed in Tables 12 and 13) and were more sensitive to storage (Table 13), groups could not clearly be differentiated on the basis of their iron-reducing ability.

The electron transport and iron reduction system in isolate Ps 200 is complex; Arnold et al. (1986a) found two reductase systems, one constitutive and the other induced

Table 14. Comparison of iron reduction rates of groups of *A. putrefaciens* strains isolated from oil field fluids

Isolate	Iron Reduction Rate @ 30°C [a.] (mg Fe^{2+}/l /h /mg protein)	Average
GROUP 1		
Ps 200	156	
Pem 12	234	
216	94	
230	160	
ATCC 8071	132	155 ± 51
GROUP 2		
213	118	
CL70	184	
ESSO 4-1	167	
ESSO 1-1	213	
ESSO 4-2	153	167 ± 35
GROUP 3		
ESSO 1-3	237	-
GROUP 4		
T2Fed 3	95	
CL41	92	
CL31	177	
CL71	106	
AN22	145	
AN24	42	
Fed 75	140	
Fed 80	75	109 ± 43

a. Reduction rate data for increasing protein concentrations and rate calculations for each isolate are listed in Appendix F. In all cases, the reaction volume was 10 ml so that iron reduction activity expressed as µmol Fe^{2+}/h/mg protein can be calculated by dividing by a factor of 5.58

by growth under low oxygen tension. The cells in the present assay were grown aerobically and should form the constitutive iron reductase system in iron-containing medium. This assay would initially measure the constitutive system, as shown by the immediate reduction of ferric iron. The effects of the induced system on this assay were not investigated.

No attempt was made to study the reaction mechanism in the various strains. This assay would be useful for any further study because rates were normalized to cell protein concentration rather that OD values as was done by Arnold et al. (1986a). It could also be adapted to compare the effects of metabolic inhibitors and the induction and competition between the various reductase systems (TMAO, $SO_3^=$, Fe^{3+} and NO_3^-) present in these isolates. The induced iron reductase system should be characterized to compare it to the inducible TMAO reductase and nitrate reductase (Easter et al. 1983; Stenberg et al. 1984) and determine the substrates used by each system.

Iron reduction by coryneform AN13

One isolate, coryneform AN13, grew to a higher OD in peptone-yeast extract based iron-free B10 medium than in modified Butlin's medium. The optimum temperature for growth and iron reduction for this organism was 37°C, and it reduced iron in B10 medium but not in the defined assay using lactate as a carbon source.

Resting cells of AN13 reduced ferric iron in B10 medium at 37°C at a rate of 106 mg Fe^{2+}/l/h/mg protein with no decrease in reduction activity as a result of storage (Table 15). This rate was slightly lower than the rates measured on Day 0 at 30°C for Esso 1-3, Ps 200 and ATCC 8071 in the same assay. For comparison of these isolates under defined assay conditions, the range of electron donors used by AN13 would have to be determined.

These results illustrate the problems of comparing different isolates under defined

Table 15. Comparison of iron reduction rates of resting cells of *A. putrefaciens* strains and coryneform AN13 in B10 medium

Temp.	Isolate	Storage time (days)	Protein concentration (µg/ml)	Iron reduction rate mg Fe^{2+}/l /h /ml cells	/mg protein [a.]
37°C	AN13 [a.]	0	- [b.]	52.1	106
		2	492	52.4	107
30°C	ESSO 1-3	0	527	152	288
		2	570	56	99
	Ps 200	0	594	181	304
	A. putrefaciens ATCC 8071	0	443	102	230

a. In all cases, the reaction volume was 10 ml so that iron reduction activity expressed as µmol Fe^{2+}/h/mg protein can be calculated by dividing by a factor of 5.58

b. AN 13 cells grown in B10 medium without $FePO_4$ instead of Bultlin's medium

c. - = not available, Day 2 protein content used for calculation of Day 0 rate

conditions and comparing the results of different studies. All the *A. putrefaciens* strains tested had growth conditions, optimum temperatures and iron reduction rates similar to those of isolate Ps 200, but other iron-reducing organisms may have different growth requirements for iron reduction. For example, Arnold et al. (1986a) compared iron reduction of isolate Ps 200 to other species using an assay based on lactate as electron donor and growth in modified Butlin's medium. This method gave significantly higher results for isolate Ps 200, as conditions were optimized for this species, but they may not have been optimal for the other isolates tested. In addition, the presence of ligands and iron speciation greatly affected the rate of iron reduction in isolate Ps 200 (Arnold et al. 1986b). Therefore, results obtained using different assay procedures with insoluble ferric iron compounds or soluble ferric iron or by employing various ligands such as nitrilotriacetic acid (NTA) (Arnold et al. 1986a) cannot be compared. The soluble ferric iron is more rapidly reduced than the solid forms (Arnold et al. 1986b). This explains why reduction rates determined for isolate Ps 200 on soluble ferric iron under optimum conditions (C.O. Obuekwe, Ph.D. thesis) appear to be higher than reduction rates for other organisms on Fe_2O_3(s) (Munch and Ottow 1983; Ottow 1968).

Reduction of insoluble ferric iron oxides

Isolate Ps 200 was able to produce ferrous ions from seven amorphous or crystalline ferric iron-containing compounds, although at very much reduced rates than from soluble $FePO_4$ (Table 16). Ferrous iron production was first tested in iron-free B10 medium which was used for the initial isolation of the organisms and which had a similar composition to other test media (Ottow 1970; Pfanneberg and Fischer 1984). The amount of ferrous iron released was similar when assayed in Butlin's medium with lactate as the energy source (Table 16).

There were no obvious changes in appearance in the inoculated oxide tubes other

Table 16. Ferrous iron release from ferric iron oxides in different media by iron-reducing *A. putrefaciens* isolate Ps 200

Iron oxide	Formula	mg Fe^{2+}/l produced in 7 days	
		Iron-free B10 [a.]	Modified Butlin's
Synthetic powder			
Ferric oxide (red)	Fe_2O_3	11.6 ± 1.8	24.9 ± 0.6
Ferri/ferrous hydroxide (black)	Fe_3O_4	14.7 ± 4.0	16.8 ± 2.9
Minerals			
Limonite	$FeOOH \cdot nH_2O$	25.3 ± 2.8	14.8 ± 0.4
Goethite	αFeOOH	14.9 ± 2.0	13.5 ± 3.2
Hematite	αFe_2O_3	8.3 ± 2.0	6.4 ± 0.4
Magnetite	αFe_3O_4	4.9 ± 0.7	4.0 ± 1.4
Siderite	$FeCO_3$	13.4 ± 5.7	14.7 ± 2.3
Ferric phosphate (soluble)	$FePO_4$	436 ± 20	nd [b.]

a. at 7 days, average pH = 6.8

b. nd= not determined

than a slight increase in turbidity. The inoculated tubes had an average pH of 6.8 after 7 days of incubation, while the mineral controls were pH 7.0. Minerals showed negligible Fe^{2+} production in the absence of inoculum. The insoluble oxides in inoculated tubes tended to lump together in a mass and microscopic observations showed some bacteria in close contact with the oxide particles. Studies by Munch and Ottow (1983) and Arnold et al. (1986b) indicate that cell contact is required for reduction of solid ferric iron.

A time course of Fe^{2+} production by isolate Ps 200 from 5 ferric oxides in Butlin's medium is shown in Figure 10. Iron was released at low rates which were dependent on the oxide form used, as reported in studies by Munch and Ottow (1983, 1980), Ottow (1969a) and Fischer and Pfanneberg (1984). Crystalline forms such as hematite and magnetite are more slowly reduced than goethite and the amorphous oxides. After 10 days, Fe^{2+} concentrations levelled off for all the insoluble oxides, except hematite, which increased steadily up to 21 days. A similar time course of solubilization was obtained in iron-free B10 medium.

The 7 isolates selected, including *A. putrefaciens* ATCC 8071, were also able to release ferrous ions from insoluble red ferric oxide at approximately equal rates: 16.5 ± 2.9 mg/l in 5 days (Table 17). The rate of release from ferric oxide was measured in days whereas the rate for soluble $FePO_4$ is much faster and was therefore measured in hours. All of the iron-reducing cultures which were isolated in B10 medium and evaluated using assays involving soluble ferric iron could also play an important role in the reduction of insoluble oxides. In addition, *A. putrefaciens* isolates should be detected by any assay procedure used to isolate iron-reducing bacteria which is based on insoluble oxides (Ottow 1968, 1969b; Ottow and Glathe 1971; Jones 1983; Fischer and Pfanneberg 1984).

Reduction rates of isolate Ps 200 on Fe_2O_3 (s) were in the same range as the highest rates reported by Ottow (1968): 25 mg/l in 5 days by *Bacillus circulans*. *A.*

Figure 10. Ferrous iron release from insoluble iron oxides in modified Butlin's medium by iron-reducing *A. putrefaciens* isolate Ps 200.

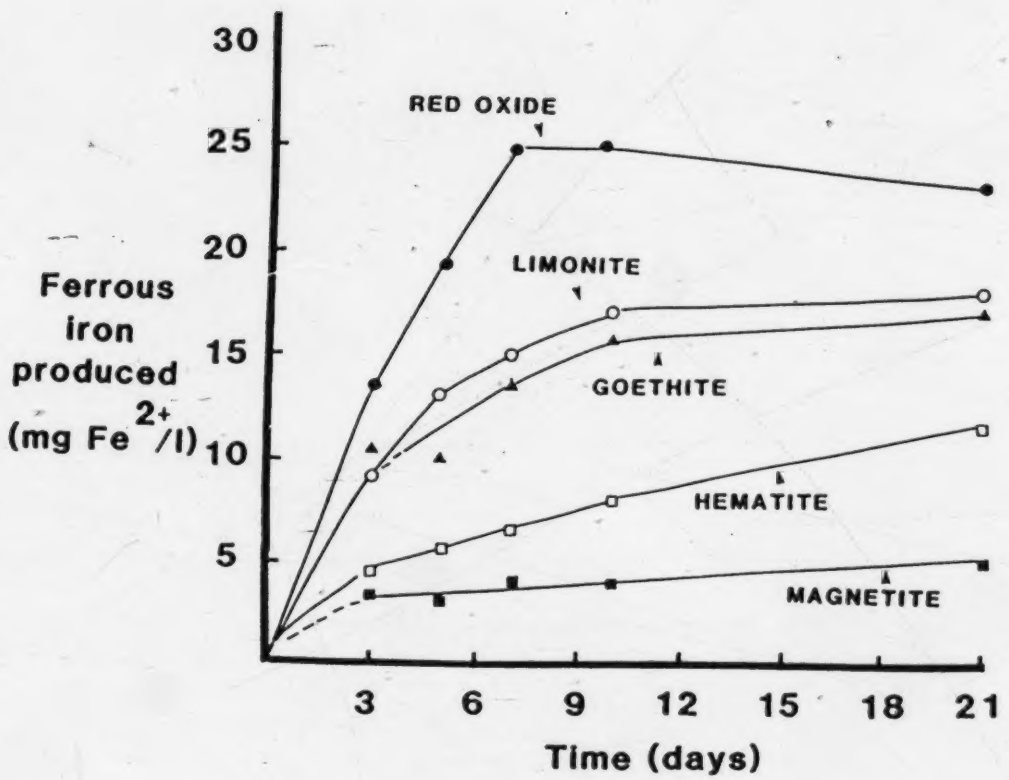

30
25
20
15
10
5
Ferrous iron produced (mg Fe 2+ /l)
RED OXIDE
LIMONITE
GOETHITE
HEMATITE
MAGNETITE
3
6
9
12
15
18
21
Time (days)

Table 17. Release of ferrous ions from powdered red Fe_2O_3 (Fisher) in modified Butlin's medium by strains of *A. putrefaciens*

Isolate	Ferrous ions produced from insoluble Fe_2O_3 (mg Fe^{2+}/l)		
	Day 5	Day 7	Day 10
A. putrefaciens ATCC 8071	19.3±0.7	24.9±0.6	25.0±1.4
Ps 200	21.3±0.7	27.0±3.1	27.6±3.5
Pem 12	17.8±2.0	14.2±5.4	17.1±0.9
CL41	13.6±0.4	13.5±0.8	13.5±3.2
T2Fed 3	14.5±0.4	14.0±0.4	12.7±0.7
ESSO 4-1	14.2±0.4	14.2±1.0	12.8±1.5
ESSO 1-1	15.0±1.0	14.9±1.2	13.3±0.7

putrefaciens strains should still be compared to other iron-reducing bacteria on insoluble oxides, under similar conditions, before statements on their iron-reducing abilities can be made.

D. SULFUR ISOTOPE FRACTIONATION DURING SULFITE REDUCTION

Anaerobic growth and sulfide production

Aerobic growth curves in modified Butlin's medium of isolates Ps 200 and Pem 12 representing group 1, and isolates T2Fed 3 (referred to as Fed 3) and CL41 from group 4, were shown in Figure 8. During both aerobic growth, and anaerobic growth with sulfite, the pH of the culture medium rose steadily to 8.8. Cells were motile when grown under aerobic conditions and during anaerobic growth with sulfite. Only isolate Ps 200 was nonmotile during the linear phase of growth with sulfite, but regained motility in the stationary phase after approximately 60% of the sulfite had been reduced. Growth with sulfite had no affect on the cell morphology of the isolates. McCready et al. (1976) reported changes in the morphology of *C. pasteurianum* in media containing high sulfite concentrations.

The data showing the anaerobic growth and sulfide production during sulfite reduction by the four oil field strains of *A. putrefaciens* (Ps 200, Pem 12, Fed 3 and CL41) are presented in Figure 11. The isolates varied in the length of time before linear growth started, in the rate of growth and in the final optical density reached. Isolate Ps 200 showed the fastest growth rate and reached a higher optical density than did the other isolates. The other isolates had varying growth rates but yielded similar final optical densities. The rates of sulfide production varied amongst the cultures studied (Table 18), with CL41 and Ps 200 having the highest rates. The total amount of sulfide generated was similar for all four isolates (Figure 11), and ranged from 77.5-85.2% of the theoretical sulfide production (Table 18). As for other dissimilatory sulfate- and

Figure 11. Anaerobic growth and sulfide production patterns during sulfite reduction by four strains of *A. putrefaciens* isolated from oil field fluids.
(a = Ps 200; b = CL 41; c = Fed 3; d = Pem 12)

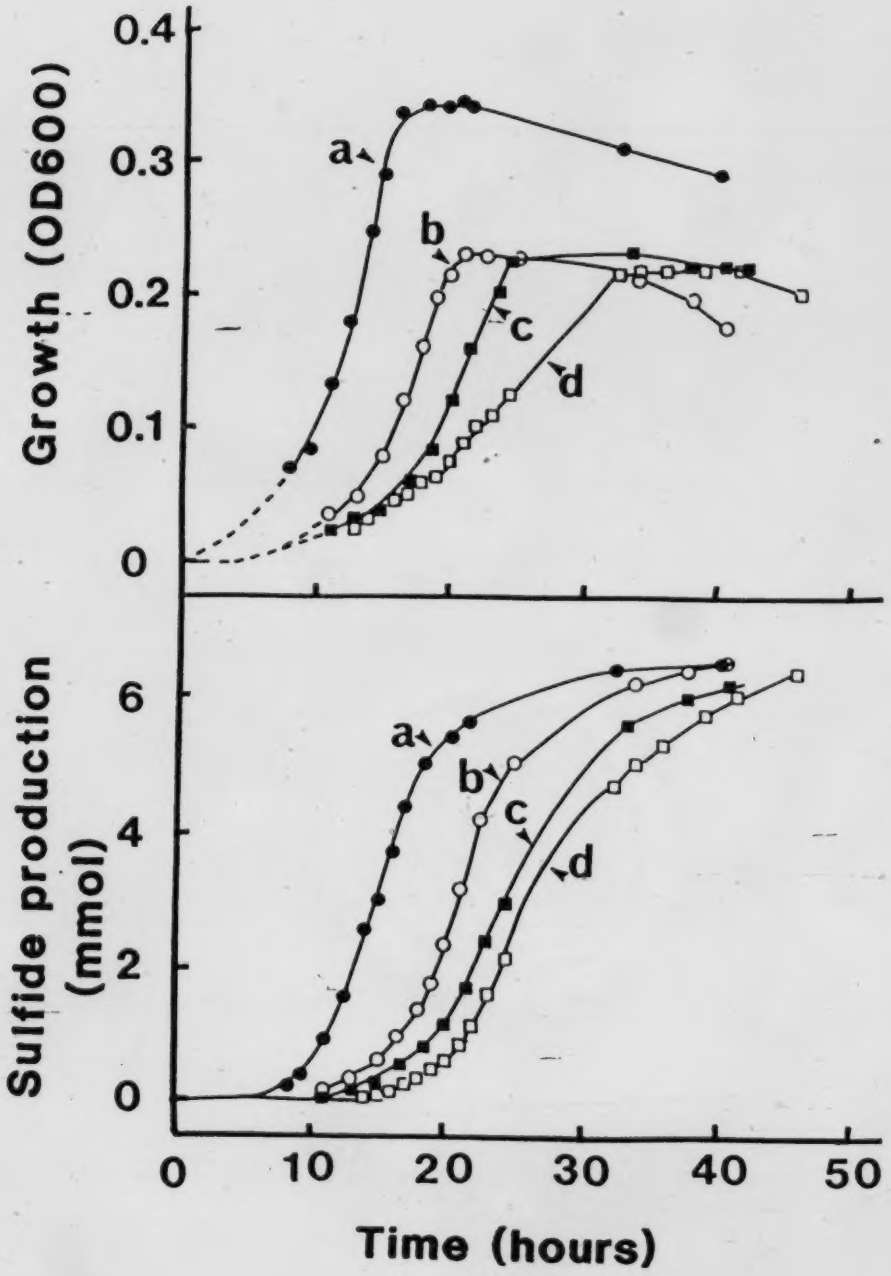

Growth (OD600)
0.4
0.3
0.2
0.1
0
a
b
c
d
Sulfide production (mmol)
6
4
2
0
a
b
c
d
0
10
20
30
40
50
Time (hours)

Table 18. Comparison of sulfide generation and sulfur isotope fractionation during sulfite reduction by four strains of *A. putrefaciens* isolated from oil field fluids

Isolate	Rate of sulfide generation (mmol/h)	Total % conversion of $SO_3^=$ to $S^=$	$\delta^{34}S$ final reservoir sulfur[a.]	$\delta^{34}S$ H_2S [b.]	
				Minimum	Maximum
Ps 200	0.60	83.3	+18.3	-14.2	+11.2
CL 41	0.70	85.2	+24.4	-14.0	+11.7
Fed 3	0.41	77.5	+14.8	-13.3	+20.2
Pem 12	0.34	83.5	+19.1	-13.6	+34.2

a. determined from $BaSO_4$ precipitate

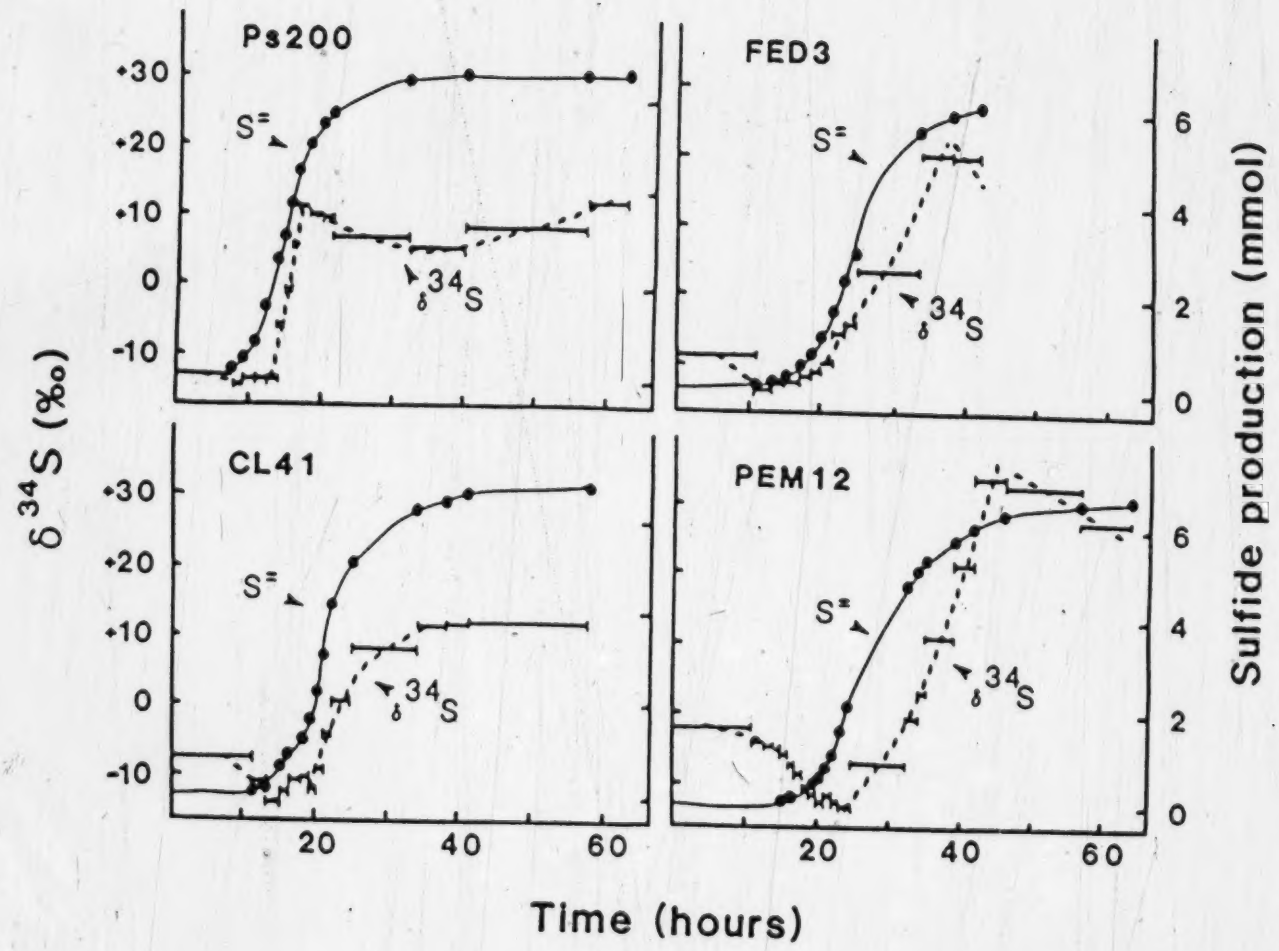
b. the $\delta^{34}S$ of the starting $SO_3^=$ was -0.1 ‰

sulfite-reducing bacteria, sulfide production paralleled growth and the maximum rate of production in *Alteromonas* spp. was observed during the linear phase of growth. This information suggests that these organisms could add significantly to the total production of H_2S in the oil field environment, depending on the availability of sulfite and a suitable carbon sources. A possible source of sulfite could result from the growth of dissimilatory sulfate-reducers. Jobson (Ph.D. thesis) reported that *Desulfovibrio vulgaris* var. *oxamicus* excreted considerable amounts of sulfite when grown at low dilution rates.

Sulfur isotope fractionation patterns

The patterns of sulfur isotope fractionation during sulfide generation, as a function of time, are presented in Figure 12. The lowest $\delta^{34}S$ values observed ranged from -13.3 to -14.2‰ (Table 18). The $\delta^{34}S$ value of the initial sulfite was determined to be -0.1‰. Maximum enrichment of ^{32}S in the sulfide produced (i.e. the normal kinetic isotope effect) occurred during the initial stages of growth and sulfide production. As the cultures entered the period of maximum sulfide production, the sulfide produced became enriched in the ^{34}S isotope. The $\delta^{34}S$ values peaked upon entry into the stationary growth phase and the rate of sulfide production decreased. The greatest $\delta^{34}S$ value was observed for the slowest growing organism, isolate Pem 12 (Figure 12). There was no further change in these values for isolate CL41, whereas they decreased for Fed 3 and Pem 12. With the Ps 200 culture, this decrease was followed by a further increase late in the stationary phase of growth. Similar patterns of isotope fractionation during sulfide production, with inverse kinetic isotope enrichment, have previously been reported for *C. pasteurianum* (McCready et al. 1975), *Salmonella heidelberg* (McCready and Krouse 1979) and for

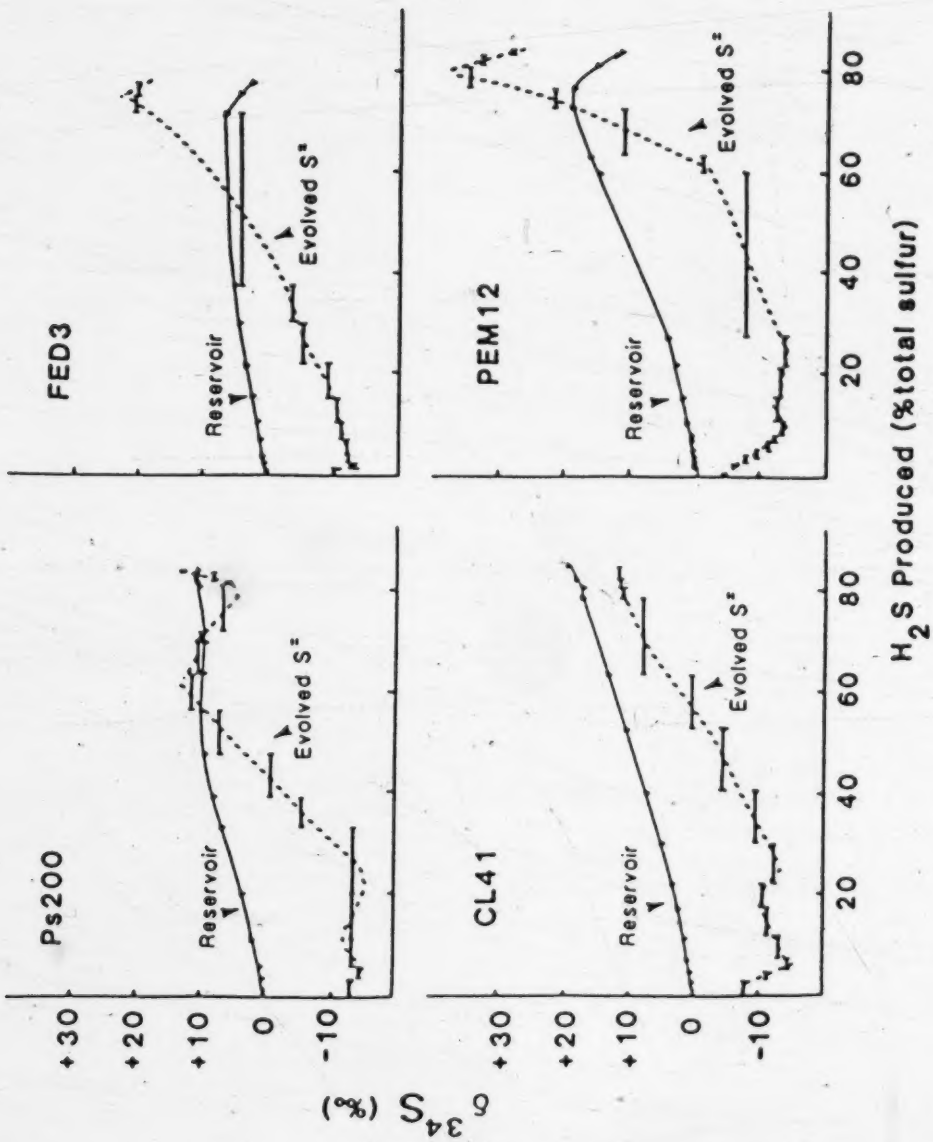
Figure 12. Sulfide production and sulfur isotope composition of the sulfide evolved during sulfite reduction by four strains of *A. putrefaciens* as a function of time. Horizontal bars correspond to the time intervals of H_2S collection.


Ps200
FED3
CL41
PEM12
S=
δ34S
+30
+20
+10
0
-10
6
4
2
0
20
40
60
δ34S (‰)
Sulfide production (mmol)
Time (hours)

other clostridial species (Laishley et al. 1984). The $\delta^{34}S$ values of the reservoir sulfur are also shown in Table 18 and as expected, residual sulfur remaining in the reservoir at the end of each run is greatly enriched in ^{34}S.

Isotope fractionation patterns as a function of the amount of sulfide produced, based on the total sulfite available, are presented in Figure 13. Comparison of the $\delta^{34}S$ values of the evolved sulfide and the calculated reservoir sulfur shows the inverse isotope fractionation effect for isolates Ps 200, Pem 12 and Fed 3. The maximum enrichment of sulfide with ^{34}S is listed in Table 18, and occurred at approximately 64% conversion for isolate Ps 200 ($\delta^{34}S$ = +11.2), 75% for isolate Fed 3 ($\delta^{34}S$ = +20.2) and 81% for isolate Pem 12 ($\delta^{34}S$ = +34.2). The sulfide produced by the latter two isolates, and in particular Pem 12 (Figure 13), subsequently became enriched in the ^{32}S isotope. Similar fractionation patterns were observed by Laishley et al. (1984) for *Clostridium acetobutylicum*, *Clostridium butyricum*, *C. pasteurianum* and *Clostridium perfringens*. Isolate CL41 (Figure 13) showed a similar pattern but no inverse isotope fractionation was realized (the $\delta^{34}S$ of the evolved sulfide was not more positive than the reservoir sulfur) and the $\delta^{34}S$ value did not decrease again. The isotope enrichment pattern of isolate Ps 200 (Figure 13) was different in that, after reaching a maximum enrichment in ^{34}S at 64% conversion, newly formed sulfide became depleted in ^{34}S (i.e. enriched in ^{32}S) until 81% conversion. Then the fractionation pattern was again reversed and the sulfide produced became enriched in ^{34}S. This type of fractionation pattern was reported by Laishley et al. (1984) for *Clostridium bifermentans*. The isotope fractionation patterns for all of the *A. putrefaciens* isolates tested are predictable and follow the trends described for *Clostridium* spp. The growth pattern, that is, the length of the early growth stage, sulfide production rate and the extent of conversion of sulfite before entry into stationary

Figure 13. Sulfur isotope fractionation patterns of sulfide evolved during sulfite reduction by four strains of *A. putrefaciens*, relative to the amount of sulfide produced. Horizontal bars correspond to the $\delta^{34}S$ value of the intervals of H_2S collected. Inverse isotope fractionation is evident when the $\delta^{34}S$ value of the evolved sulfide is higher than the value calculated for the remaining reservoir sulfur.


FED3
PEM12
Ps200
CL41
Reservoir
Evolved S²
+30
+20
+10
0
-10
20
40
60
80
$\delta^{34}S$ (‰)
H_2S Produced (%total sulfur)

phase, influences the overall magnitude of the inverse isotope effect observed.

Summary

These results of the sulfur isotope studies with *A. putrefaciens* strains have been published (Semple and Westlake 1987b). The data reported indicate that strains of *A. putrefaciens* isolated from oil field fluids can readily produce sulfide and carry out sulfur isotope fractionation during anaerobic growth with sulfite as the terminal electron acceptor. The isotope fractionation patterns observed for these bacteria are similar to those previously reported for clostridial species, and they provide further support for the use of isotope fractionation studies for the diagnosis of dissimilatory sulfite reduction. These organisms could contribute to the production of H_2S in the oil reservoir and thus, the overall fractionation of sulfur isotopes observed.

E. CORROSION BY PURE AND MIXED CULTURES OF IRON-REDUCING BACTERIA AND SULFATE-REDUCING BACTERIA

The weight loss measured and the calculated corrosion rates of mild steel corrosion coupons exposed to pure and mixed cutures of *A. putrefaciens* isolate Ps 200 and *D. vulgaris* AL1, in different media, are presented in Table 19. The weight loss measurements indicate whether a significant amount of general corrosion attack has taken place and are measured after acid removal of all the corrosion products and attached material from the coupons. In order to evalute the microbial corrosion process, the visual and microscopic observations of the changes in the coupons must also be considered. These observations include a description of the extent of biofilm formation and the type and the amount of corrosion product and biofilm that was formed. This can involve the determination of the weight gain measurements if appreciable corrosion product remains intact and adhering to the coupons. The appearance of the coupons as the biofouling is

Table 19. Corrosion rates of mild steel coupons in mixed and pure cultures of isolates *A. putrefaciens* Ps 200 and *D. vulgaris* AL1 in different media

Corrosion Test Medium and inoculum	Time (weeks)	Weight loss mg [a.]	Weight loss mg/dm^2 [b.]	Calculated corrosion rate mdd [c.]	Calculated corrosion rate mpy [d.]
Butlin's					
Control	3	24.8 ± 6.3	210	10.0 ± 2.5	1.8
	6	32.2 ± 0.9	273	6.5 ± 0.5	1.2
	12	37.9 ± 0.2	321	3.8 ± 0.1	0.70
Ps 200	3	9.8 ± 0.2	83	4.0 ± 0.1	0.72
	6	12.9 ± 0.6	109	2.6 ± 0.1	0.48
	12	20.7 ± 1.1	176	2.1 ± 0.1	0.38
AL1	6	57.3 ± 1.9	486	11.6 ± 0.4	2.1
	12	32.9 ± 2.4	279	3.3 ± 0.2	0.61
Mixed	6	8.5 ± 1.7	72	1.7 ± 0.3	0.31
	12	11.3 ± 0.7	96	1.1 ± 0.1	0.21
Butlin's -YE					
Control	12	42.7 ± 1.9	362	4.3 ± 0.2	0.79
Ps 200	12	45.7 ± 1.7	388	4.6 ± 0.2	0.84
AL1	12	184.4 ± 10.7	1564	18.6 ± 1.1	3.4
Mixed	12	224.5 ± 12.7	1904	22.7 ± 1.3	4.2
Butlin's +$S_2O_3^=$					
Control	12	90.0 ± 10.0	763	9.1 ± 1.0	1.7
Ps 200	12	19.7 ± 1.3	167	2.0 ± 0.1	0.36
AL1	12	148.9 ± 12.7	1263	15.0 ± 1.3	2.8
Mixed	12	35.4 ± 7.4	300	3.6 ± 0.7	0.65

a. Weight loss and standard deviation determined from triplicate specimens. Weight loss due to acid treatment alone, 1.0±0.1 mg, was subtracted from each value
b. mg/dm^2 = weight loss per unit area. Area of coupons = 0.1179 dm^2
c. mdd = mg/dm^2/day
d. mpy = mils per year = mdd x 1.44/ density. Density of mild steel = 7.86 g/cm2

removed during rinsing, sonication and acid treatment, and evaluation of the pitting and localized corrosion seen, must also be considered in relation to the weight loss measured.

The culture pH and final cell counts in the medium at the end of each test period are shown in Table 20. There was no significant change in pH in any of the tests with the final pH ranging from 6.5 to 7.5. The counts given represent the number of cells suspended in the medium (i.e. planktonic) and not the bacteria attached to the coupons (i.e. sessile) and indicate that there are still viable cells in the cultures. Observations of the significant changes in culture appearance and the development of biofouling on the coupons during the course of the experiments will also be discussed for each test.

Corrosion of sterile controls

Under the experimental conditions used, that is, with stirring and no artifically created anaerobic conditions, the controls would have remained aerobic. No 'anaerobic' controls were established in this series of experiments as they were modeled after Obuekwe's (Ph.D. thesis) experiments, and would have created another variable for the inoculated tests. Relatively high weight loss was found for control coupons after 3 weeks, resulting in an initial corrosion rate that was high, but which slowed down markedly between 6 and 12 weeks incubation (Table 19). This indicates that the coupons corroded rapidly, undergoing extensive oxidation which resulted in the development of a passivating oxide coating and a retardation of the corrosion process. The control coupons developed an even, heavy coating of yellow-orange, amorphous crystals within 3 days in all the media (Figure 14a). The amount of this yellow, 'spongy' coating did not increase beyond the first week, did not reform again if disrupted, and could be scraped off easily with tweezers during handling. A thick, cracked, dark brown, more protective oxide layer was exposed below this amorphous yellow coat after various amounts had been removed by sonication (Figure 14b). Patches of exposed, bare metal could also be seen beneath this cracked, bubbly, oxide layer.

Table 20. Final cell number and pH of mixed and pure cultures of isolates *A. putrefaciens* Ps 200 and *D. vulgaris* AL1 in different media during corrosion experiments

Corrosion Test			Viable cell number /ml culture [a.]	
Medium and Inoculum	Sample time (weeks)	pH	Plate counts Ps 200	MPN *D. vulgaris* AL1
Butlin's				
Control	3	7.0	- [b.]	-
	6	6.9	-	-
	12	7.0	-	-
Ps 200	3	6.7	$9.8 (\pm 1.8) \times 10^7$	-
	6	6.6	$3.2 (\pm 0.4) \times 10^9$	-
	12	6.5	$9.0 (\pm 1.3) \times 10^7$	-
AL1	6	7.1	-	1.00×10^0
	12	6.9	-	7.90×10^6
Mixed	6	7.0	$1.77 (\pm 1.9) \times 10^8$	9.50×10^6
	12	7.1	$8.3 (\pm 1.4) \times 10^7$	3.50×10^8
Butlin's - YE				
Control	12	7.2	-	-
Ps 200	12	6.8	$1.18 (\pm 0.08) \times 10^8$	-
AL1	12	7.2	-	1.30×10^6
Mixed	12	7.3	$6.8 (\pm 0.9) \times 10^7$	3.30×10^7
Butlin's + $S_2O_3^=$				
Control	3	7.5	-	-
	6	nd [c.]	contaminated	-
	12	7.0	-	-
Ps 200	3	6.7	$1.54 (\pm 0.26) \times 10^8$	-
	6	6.6	$1.13 (\pm 0.15) \times 10^8$	-
	12	6.7	$5.0 (\pm 0.5) \times 10^7$	-
AL1	12	7.0	-	6.40×10^5
Mixed	12	7.0	$<1.0 \times 10^4$	2.80×10^5

a. Average counts for the inocula were $4.0 (\pm 2.3) \times 10^9$/ml Ps 200 and $4.2 (\pm 4.3) \times 10^8$/ml *D. vulgaris* AL1 resulting in initial counts of about 8×10^7 cells/ml Ps 200 and/or 8.4×10^6 cells/ml AL1 for each 500 ml test flask.

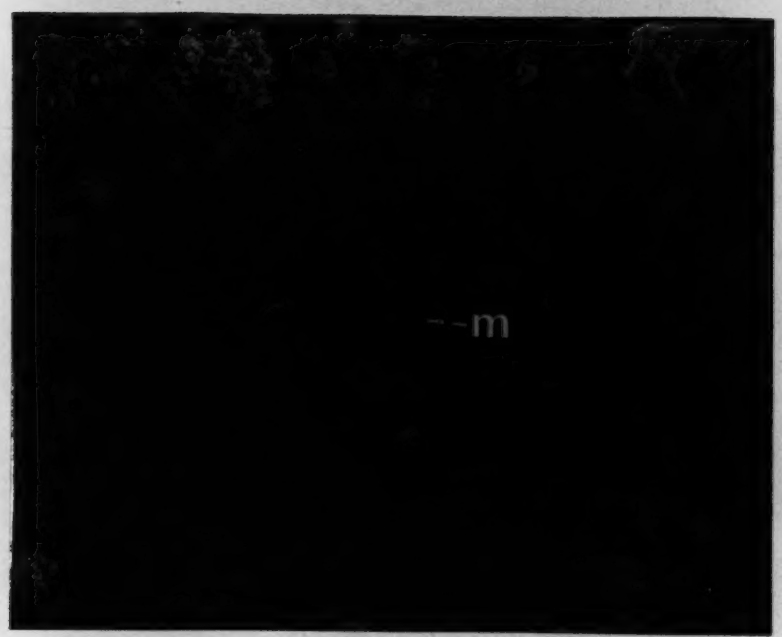
b. - = not present

c. nd= not determined

Figure 14. Scanning electron micrographs of the surface coating on a mild steel control coupon after exposure for 12 weeks in continuous culture in Butlin's medium.
A. Shows the amorphous nature of the yellow-orange coating which formed on control coupons in the three media tested and
B. After sonication, showing the thick, cracked oxide layer beneath, with patches of exposed metal surface (m). Size bars are included in the margin of all photographs for reference.

A

B



Sterile conditions were maintained in the controls throughout the experiments, with the exception of the 6 weeks control $+S_2O_3^=$, which became contaminated after 3 weeks. The contaminant did not produce sulfide or affect the coating which had already formed.

Corrosion rates for controls after 12 weeks incubation were about the same in Butlin's medium (0.70 mpy) and Butlin's -YE (0.79 mpy), whereas thiosulfate was more corrosive and resulted in higher corrosion rates, about double the other controls (1.66 mpy). Similar yellow coatings formed on all coupons in the different media but in Butlin's $+S_2O_3^=$ blackened spots developed in the coating and around the washer. The undercoating beneath the yellow coat was much darker and thicker with thiosulfate present than for the other controls but was similar in appearance to the cracked, protective oxide seen by SEM examination (Figure 14b). X-ray analysis of the blackened spots showed that the blackening was not due to iron sulfide because the coating contained only iron, phosphorus, and chlorine.

Acid treatment with Clarke's solution removed all of the oxide coating from the control coupons, leaving a even, gray metal surface. No obvious corrosion or surface changes were evident, suggesting a uniform, general corrosion had occurred. However, the control coupons from Butlin's $+S_2O_3^=$ had a more mottled, tarnished appearance than the other controls, again indicating the corrosiveness of the thiosulfate. Thiosulfate has been found to promote pitting of stainless steel in $SO_4^=/S_2O_3^=$ solutions (Garner 1985; Newman 1985; Newman et al. 1986).

Acid cleaning of the coupons

A one minute acid treatment with Clarke's solution was very effective in removing adherent corrosion products from the surface of the coupons to leave bare metal and further treatments were usually not required. Fresh coupons were silver in color with a

rough surface due to the sand blasting treatment. The very rough, flakey, convoluted surface of a fresh coupon after fixing and after acid treatment is shown in Figure 15a. Acid removed the coatings, but did not change the overall appearance of the coupons or affect the crystal structure so that the changes in surface roughness or structure of corroded test coupons could be evaluated. Acid cleaning of fresh, untreated coupons for one minute resulted in a weight loss of 0.0010 ± 0.0001 grams. This value was subtracted from all weight loss determinations for the calculation of the corrosion rates of controls and inoculated tests. The initial weight of the corrosion coupons was between 5.9 and 6.2 grams.

Corrosion and attachment by *A. putrefaciens* isolate Ps 200

a. Growth and attachment in Butlin's medium: Corrosion rates found with pure cultures of *A. putrefaciens* isolate Ps 200 were generally lower than for the controls, which was also noted by Obuekwe (Ph.D. thesis). The culture flasks were very cloudy and the organisms grew well as noted by the high counts of organisms suspended in medium (Table 20). The coupons remained clean and essentially free of coating throughout the 12 weeks incubation period, showing no build up of the yellow product that occurred in the controls. The extensive oxidation of the metal coupons that was seen in the controls would not be likely in the inoculated tests because the growth of isolate Ps 200 would quickly create anaerobic conditions. In this experiment, the iron-reducing isolate Ps 200 did not remove the coating from the coupons, as suggested by Obuekwe et al. (1981b), but probably prevented its formation by removing oxygen.

Observation of the coupons from Butlin's medium under SEM after fixing and drying showed that only a thin surface coating had formed (Figure 15b). There was very little attachment of the bacteria to the coupons in this medium, and rod-shaped bacteria covered with crystalline material were visible in the bare spots, dispersed sparsely and

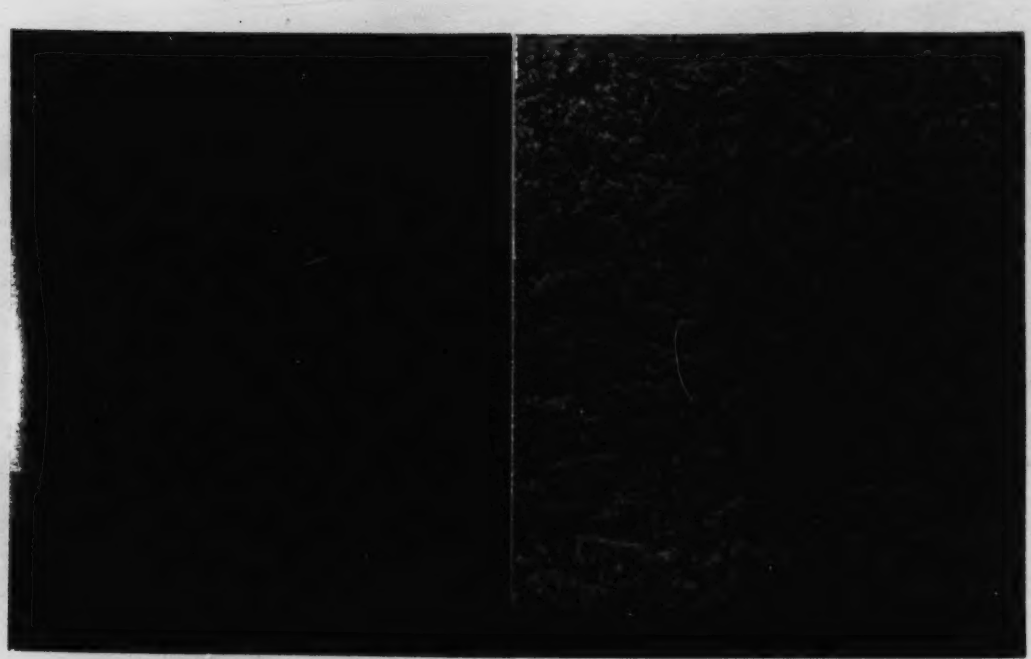
Figure 15. Comparison of the surface coating on mild steel coupons before and after exposure for 12 weeks in continuous culture to *A. putrefaciens* isolate Ps 200 in different media.

A. Surface of a sand-blasted, unexposed mild steel coupon, after fixing and acid treatment. Note the rough, flakey nature of the metal surface.

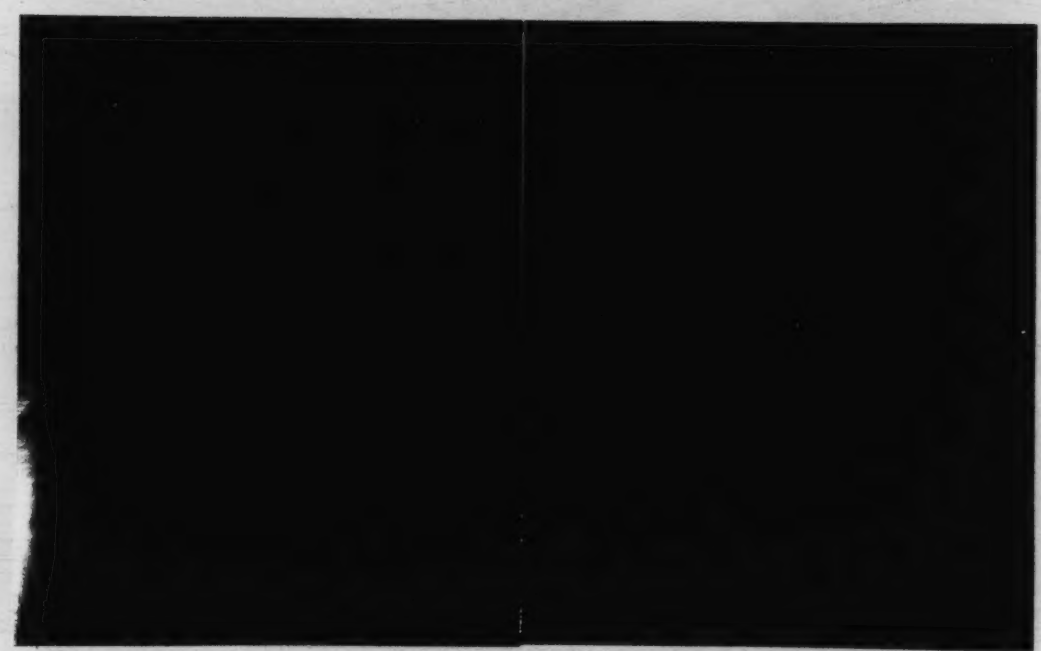
B. Surface coating in Butlin's medium.

C. Thin, black iron sulfide surface coating from Butlin's $+S_2O_3^=$

D. Crystalline, blue surface coat on coupons from Butlin's -YE.



A B



C D

evenly over the surface on the 3 week coupons (Figure 16 a,b). There was no increase in attachment after 6 or 12 weeks incubation, but the 12 week coupon appeared to have more crystals and oxide than did the 3 week old coupons. X-ray analysis of some this material on the 12 week coupons showed the presence of iron, phosphorus with some potassium and calcium. Oxides were likely present but oxygen is not detected by X-ray analysis. Ultrasonic cleaning of the coupons removed the attached bacteria and corrosion product, and leaving a shiny, gray, uniform surface with no obvious corrosion. Obuekwe et al. (1981b) also implied that there was poor attachment of isolate Ps 200 in Butlin's medium because they had difficulty distinguishing cells attached to coupons after 2 weeks whereas they reported good attachment of this organism in B10 medium.

b. Growth and attachment under sulfide generating conditions: When isolate Ps 200 was grown in Butlin's $+S_2O_3^=$, copious amounts of sulfide were formed, resulting in the blackening of the medium. However, the organisms did not attach well to the coupon surface and formed a protective FeS layer on the metal which inhibited corrosion and resulted in very low corrosion rates (Table 19). The black amorphous layer, composed mostly of iron and sulfur with some phosphorus, formed a very thin covering over the surface and the rough surface structure of the metal was still distinguishable (Figure 15c). There was less coating than had developed in tests with Butlin's medium but there were only slightly more bacteria attached, with some clumps and colonies of organisms visible (Figure 16 c,d). Again, there was not much difference in the amount of coating developed or the number of organisms attached between the 3 week and 12 week samples. Theoretically, isolate Ps 200 should have been able to grow anaerobically, attached to the coupons, using $S_2O_3^=$ as an electron acceptor, but again, Butlin's medium seemed to inhibit the attachment of this isolate.

Figure 16. Comparison of the amount of surface coating and the attachment of bacteria to the surface of mild steel coupons after incubation with *A. putrefaciens* isolate Ps 200 in (A, B) Butlin's medium for 3 weeks and (C,D) Butlin's $+S_2O_3^=$ for 12 weeks.

A B

C D

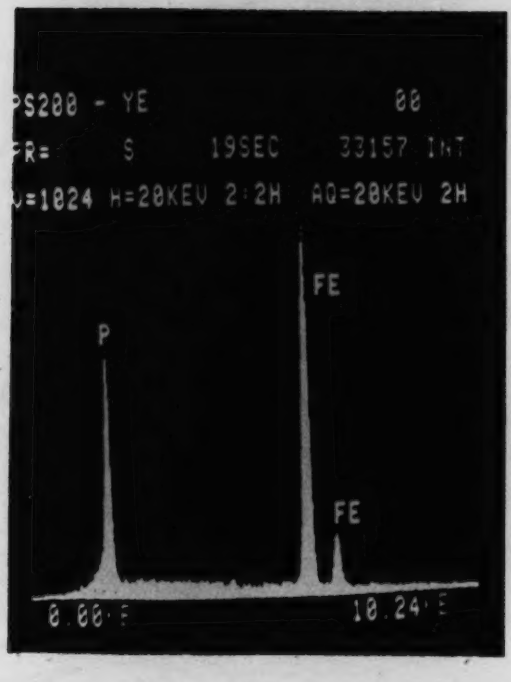
Sonication of the coupons removed the cells but a thin black film still coated the coupons. After sonication, coupons from the 6 week test were covered with patchy spots of bright clean metal which varied in size from 0.1 to 0.5 mm where this black covering had been removed. Acid removed this coating and the coupons were slightly discolored but showed no obvious corrosion or actual pitting. Obuekwe (Ph.D. thesis) also reported the formation of a thin FeS film with bright spots and 'pitting' after 9 weeks in continuous culture with isolate Ps 200 grown with thiosulfate. However, he did not indicate whether actual pits could be distinguished.

c. Growth and iron phosphate formation in Butlin's -YE: Coupons from tests with isolate Ps 200 in Butlins -YE had approximately the same corrosion rate as the control coupons and 2 times higher than rates found in Butlin's medium (Table 19). Growth in medium without yeast extract was less cloudy, and the orange color of the organisms was less intense than when yeast extract was present, but the viable counts were similar in both media (Table 20). In the -YE medium, the coupons developed an even velvety, white coating on the surface and a buildup of pinkish material, most likely organisms, on top of the washer. After fixing and drying, coupons were covered with blue crystals but no bacteria were seen by SEM (Figure 17a). After sonication, the crystals in the buffer also oxidized and turned blue. Some bacteria were found in the fluid but not on the sonicated coupons and many of the blue crystals remained on the surface. Energy dispersive X-ray spectrum analysis of the crystals showed a high concentration of iron and phosphorus (Figure 17b), so they were probably ferrous phosphate, possibly vivianite, which forms white-blue monoclinic crystals (Weast 1975). Similar blue crystals were produced by oxidizing the white precipitate which formed in B10 medium (see Iron reduction section). Iron-reducing organisms are known to form vivianite in phosphate-containing medium (C. Panter, M.Sc. thesis; Arnold et al. 1986a). Acid treatment removed these crystals and revealed a convoluted, flakey surface similar to the

Figure 17. Scanning electron micrograph (A) and energy dispersive X-ray spectrum (B) of the crystalline, blue coating which developed on the surface of mild steel coupons exposed in continuous culture to *A. putrefaciens* isolate Ps 200 in Butlin's -YE for 12 weeks. The crystals were composed of iron and large amounts of phosphorus.

A

B


S200 - YE
00
R= S 19SEC 33157 INT
=1024 H=20KEV 2 2H AQ=20KEV 2H
FE
P
FE
0.00 E
10.24 E

controls, with no obvious corrosion or changes to the crystal structure.

The bacteria may have associated more closely to the metal surface, reducing any ferric oxide coating present and forming the ferrous phosphate precipitate. Any organisms present on the surface could have been occluded by crystals. The corrosion rate in this media was now the same as the aerobic control, which indicates that the organism may have slighty enhanced corrosion of the coupons under these conditions.

Growth and attachment of *Desulfovibrio vulgaris* AL1

Coupons exposed to cultures of *D. vulgaris* AL1 showed higher corrosion rates in the three media tested, with the exception of AL1-Butlin's (12 wk), than were found in the aerobic controls (Table 19). There were some differences, however, in the development of biofouling and extent of the corrosion seen in the different media used.

a. Establishment of cultures: Even though no attempt was made to impose anaerobic conditions with deaeration or oxygen scavengers, pure cultures of strictly anaerobic sulfate-reducing bacteria were able to establish and grow in the corrosion flasks used in these experiments. Counts of SRB after 12 weeks in continuous culture ranged from 6.4×10^5 to 7.9×10^6 cells/ml. However, growth of *D. vulgaris* AL1 in pure culture was highly variable in the different media, possibly due to varying abilities of the different aliquots of inocula to poise the medium and initiate growth (Fedorak et al. 1987).

All of the flasks blackened within the first day after inoculation, but after stirring and media flow were started, the medium cleared again, as if the cultures had died off. However, blackening of the coupons and the walls of the flasks ocurred and some suspended black particles were still seen in these cultures. Growth was re-established after 2 to 4 days and the flasks again blackened in two of the test conditions (i.e.

AL1-Butlin's (6 wk) and AL1 +$S_2O_3^=$). Once the cultures were established, they went through varying cycles of sulfide production, when the flasks changed from cloudy gray to black. In the test system AL1-Butlin's (12 wk), this dieoff did not occur and the media remained black so that the suspended coupons were not visible throughout the test period.

In contrast, in Butlin's -YE, the culture of isolate AL1 required 6 weeks to re-establish growth and sulfide production. After the initial period of clearing, the coupons developed a yellow, amorphous coating after 1 week, as in the controls. However, there was some blackening of the coupons around the washer and under the amorphous coating as the organisms appeared to colonize the coupon. A steady buildup of black material was noticed between the 4th and 6th weeks until the medium blackened again and the coupons were no longer visible. This type of development of biofouling by SRB, in which blackening develops under rust, was noted in field experiments on biofouling in seawater (Starkey 1986).

The longer recovery time in this medium without yeast extract is not unexpected, since addition of yeast extract to media for enumeration of SRB increases the resulting counts and has led to the suggestion that components in yeast extract are required for growth of SRB (Pankhurst 1971; Abd-el-malek and Rizk 1958,1960). Postgate (1984) suggested that the apparent requirement for yeast extract may be a result of its ability to poise the redox potential (by cysteine) or to affect the solubility and availability of iron, since SRB have an absolute requirement for iron for growth.

b. Variations between replicate flasks: In Butlin's medium, there were notable differences in appearance between duplicate flasks and differences in the amount of biofouling observed were reflected in great variation in corrosion rates. Corrosion test coupons were expected to show an increase in weight loss with increased exposure time, as found with the other tests (Table 19). However, at 6 weeks, AL1-Butlin's coupons

showed a greater weight loss than was observed after 12 weeks incubation and a higher corrosion rate than the control.

The flask that was incubated for 12 weeks stayed black throughout the experiment. Viable counts of the SRB suspended in the medium at 12 weeks were relatively high (7.9 x 10^6/ml), with many long, motile rods found in the turbid medium. The coupons had developed a black, slimy fouling, but had less of this coating than was found on coupons from the 6 week test. In contrast, the flask for the 6 week incubation period had very low MPN counts (1x10^0/ml) (Table 20). The medium was clear and very few free floating rods were found by microscopic examination but the coupons were heavily fouled and stringy and it appeared that all growth was associated with the coupons. These results emphasize that the number of organisms suspended in the medium is not directly related to the number of bacteria attached, the development of biofouling or the severity of corrosion. Variability in the growth response of isolate *D. vulgaris* AL1 in replicates makes interpretation of this data difficult.

c. Attachment and biofouling of coupons: *D. vulgaris* AL1 readily attached to the coupons in pure culture and developed various amounts of heavy black, slimy coating under the different experimental conditions used. This coating dried into a black, amorphous crumbly, rough, uneven coat as shown in Figure 18.

The cracked coating on the AL1-Butlin's (12 wk) coupon is shown in Figure 19a. At higher magnification, this coating was a thick, heavy mat of long filimentous and spiral shaped rods interspersed with amorphous crystals of iron sulfide (Figure 19b). The coating on coupons from AL1-Butlin's (6 wk) was a black, powdery dry material that crumbled more easily and showed very dense colonies of bacteria among FeS clumps. Coupons from both the 6 and 12 week exposures had a thick attached film, but the 6 week coupons had more of this coating. *D. vulgaris* AL1 showed very good attachment

Figure 18. Comparison of the amount of surface coating on the mild steel coupons after exposure for 12 weeks in continuous culture to pure culture of *Desulfovibrio vulgaris* AL1 in different media.

Scanning electron micrographs are of fixed and dried coupons from these media include:

A. Butlin's medium

B. Butlin's +$S_2O_3^=$. The very heavy black fouling on coupons dried into a thick, cracked coating.

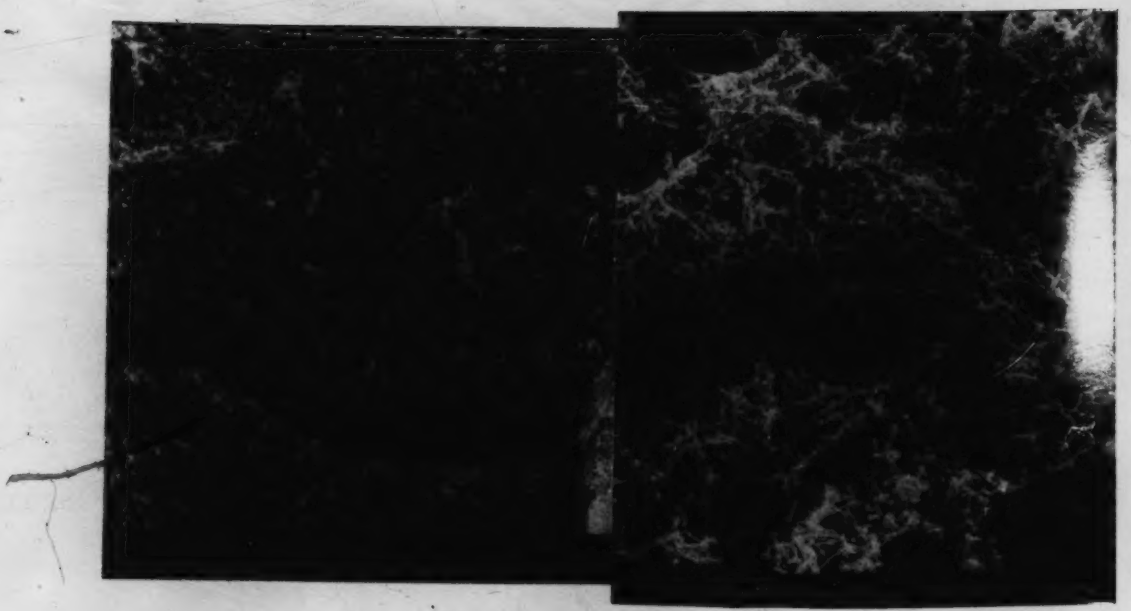
C. Butlin's -YE. Most of the attached black fouling was washed off during rinsing.

A

B C

Figure 19. Scanning electron micrographs of the surface coating attached to mild steel coupons exposed for 12 weeks to pure culture of *D. vulgaris* AL1 in (A,B) Butlin's medium and (C,D) Butlin's $+S_2O_3^{=}$.

The heavy black, slimy surface coating (A,C) on the corrosion coupons, on further magnification, was actually mats of long filamentous, rods (B,D) interspersed with amorphous iron sulfide crystals.



A B

C D

and more biofilm developed in Butlin's $+S_2O_3^=$ than in Butlin's medium alone. Coupons retrieved from this medium were covered with a very heavy, even slime which dried into a cracked coating (Figure 18b) again, composed of very long, filamentous fat rods intertwined in thick mats (Figure 19c,d). In both cases in Butlin's medium, cells of AL1 were very long, filamentous rods, even longer than found suspended in the culture medium.

Even though the biofouling on the coupons developed only in the last 6 weeks of the exposure in cultures of *D. vulgaris* AL1 in Butlin's -YE, coupons were heavily coated with a thick, black slime. This material was loosely attached and easily removed by gentle rinsing so that less corrosion product remained attached to the coupons after fixing and drying (Figure 18c). The remaining coating consisted of amorphous crystals of FeS and fewer visible organisms than were seen on coupons from the other media (Figure 20c). The medium had some affect on cell morphology, as well as growth,because cells on coupons from Butlin's -YE were smaller rods (Figure 20c) compared to the long, filamentous, spiral rods when grown with yeast extract (Figure 20 a,b). Cells growing suspended in the culture of medium -YE were also shorter than cells grown with yeast extract. Cells on the coupons from Butlin's $+S_2O_3^=$ were very long and slightly fatter than in Butlin's medium (Figure 20a); clumps of amorphous FeS and some fine extracellular material can be seen between the cells in both cases (Figure 20 a,b).

Growth and attachment in mixed culture

The highest corrosion rate found, 22.7 ± 1.3 mg dm^{-2} day^{-1} (mdd) or 4.2 mils per year (mpy), was with mixed cultures of both *A. putrefaciens* Ps 200 and *D. vulgaris* AL1 in Butlin's -YE. The corrosion results in mixed cultures were the most variable, however, with the highest and lowest corrosion rates recorded in these experiments

Figure 20. Comparison of the cell morphology of *D. vulgaris* AL1 in the surface coating attached to mild steel coupons in different media.
Note the long filamentous rods in the biofouling from (A) Butlin's medium and (B) Butlin's $+S_2O_3^{=}$. 'Strings' of extracellular material (e) can be seen between the cells. In Butlin's -YE (C), only a few, short rods (r) can be distinquished in the material that was attached to coupons.

A

B

C

(Table 19). Growth of isolate AL1 in mixed culture with aerobic isolate Ps 200 had a marked affect on the pattern of establishment of the cultures, development of biofouling and the corrosion of coupons.

a. Establishment of mixed culture: In mixed culture, high populations of both organisms were found suspended in the culture liquid, with the exception of mixed $+S_2O_3^=$ (Table 20). Growth and blackening established quickly and the clearing that was seen with pure cultures of *D. vulgaris* AL1 after stirring was started was not observed with mixed cultures. These cultures produce large amounts of sulfide, as evident from the blackening of the medium so that the coupons were no longer visible. Coupons in mixed culture developed black 'slime', as was seen in pure cultures, but were quite different in the degree and type of fouling in the different media.

b. Attachment and biofouling of coupons in different media: Corrosion rates found for the mixed culture in Butlin's medium were very low, even less than with isolate Ps 200 and the controls (Table 19). Even though there was good growth of both organisms in the medium, the coupons were not as heavily fouled (Figure 21a) as isolate AL1 alone in the same medium (Figure 18a) and had much less coating than mixed -YE (Figure 21b). A closely associated film formed on the surface, with small raised patches, which may be colonies of the organisms as seen in Figure 22a. Higher magnification showed large numbers of bacteria spread uniformly over the surface with mixed vibrio shaped and long rods and amorphous FeS clumps (Figure 22b).

Although Butlin's -YE did not support as heavy, cloudy growth with the mixed culture as did Butlin's medium, there were still high counts of both organisms suspended in the liquid (Table 20) and much more material fouled the coupons. The mixed culture

Figure 21. Comparison of the amount of surface coating on the mild steel coupons after exposure for 12 weeks in continuous culture to mixed culture of isolates *A. putrefaciens* Ps 200 and *D. vulgaris* AL1 in (A) Butlin's medium and (B) Butlin's -YE.

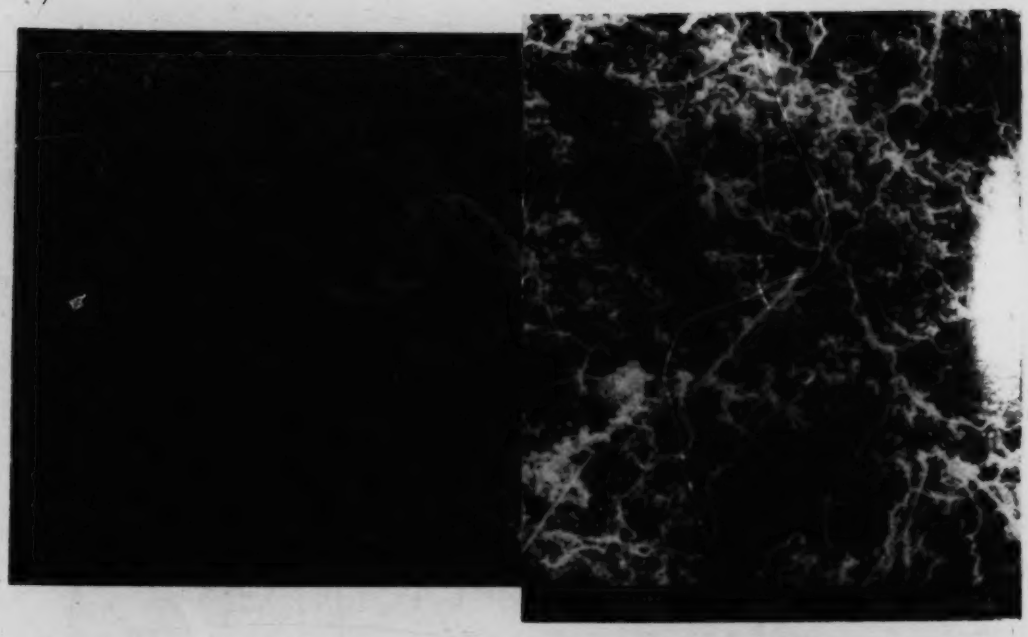
A. Coupons were not as heavily fouled in mixed culture in Butlin's medium. Note the raised patches in the coating.

B. Coupons from mixed culture -YE developed the heaviest surface coating in these experiments.

A

B

Figure 22. Scanning electron micrographs of the surface coating attached to mild steel coupons exposed for 12 weeks to mixed culture of isolates Ps 200 and *D. vulgaris* AL1 in (A,B) Butlin's medium and (C,D) Butlin's -YE. The bacteria in the coating were long filamentous and spiral rods, presumably *D. vulgaris* but there was no clear distinction between the cells of isolate Ps 200 or of AL1.



A B

C D

established much faster in this media than the pure culture of isolate AL1, which took 6 weeks to establish growth. This was likely due to removal of oxygen and reduction of the redox potential by growth of isolate Ps 200. The coupons began to develop biofouling by Day 3 and material built up steadily throughout the 12 weeks incubation period, so that they became heavily fouled with black, stringy 'slime' (Figure 23). There was even more attachment of biofouling here than with the pure culture of isolate AL1 or mixed culture in Butlin's. The heavy coating was quite hard, and could be taken off in crusty patches when the washer was removed, and was thick, black and hard when dried (Figure 21b). Mats of amorphous FeS and long filamentous rods and some tight spirals of *Desulfovibrio* were seen in the SEM photographs (Figure 22d). The difference in cell morphology of isolate AL1 in medium with or without YE were not seen in mixed culture (Figure 24). *D. vulgaris* AL1 grew better in mixed culture than in pure culture in Butlin's -YE, as the cells were longer, and there was better biofilm development. In both cases in mixed culture, SRB occurred throughout the biofouling material and were not confined to the lower reachs of the biofilm. Other than the fact that the cells of AL1 sometimes formed spirals, it is difficult to distinguish between the two organisms in order to determine their relative proportions in the biofilm.

In Butlin's $+S_2O_3^{=}$, corrosion rates with mixed culture were also very low (Table 19) because a protective FeS layer formed on the coupons. The mixed culture established quickly but changed from being initially black and turbid to clear near the end of the 12 week experimental period. There were very low numbers of isolate Ps 200 suspended in the culture liquid (below countable level) but relatively high numbers of SRB (Table 20). There appeared to be very good attachment and the coupons were heavily fouled as evident from the slimy 'fingers' of material shown in the rinsing beaker (Figure 23b). However, the heavy growth attached to the coupons was a 'filmy' grayish material, more like exopolymer, compared to the black FeS-rich fouling observed in the other tests. This

Figure 23. Comparison of the type of fouling which developed on mild steel coupons exposed to continuous culture in tests with mixed culture of isolates Ps 200 and *D. vulgaris* AL1 in (A) Butlin's -YE and in (B) Butlin's $+S_2O_3^{=}$. Contrast the heavy, black, iron sulfide rich fouling on the coupons and supports in (A) with the filmy, gray, exopolymer-type fouling in (B).

A

B

Figure 24. Comparison of cell morphology of organisms in the surface coating on mild steel coupons exposed to mixed culture of isolates Ps 200 and *D. vulgaris* AL1 and in (A) Butlin's medium and (B) Butlin's -YE. Tight spirals of *D. vulgaris* and long filamentous rods are interspersed with amorphous iron sulfides and exopolymer are found in both media.

A

B

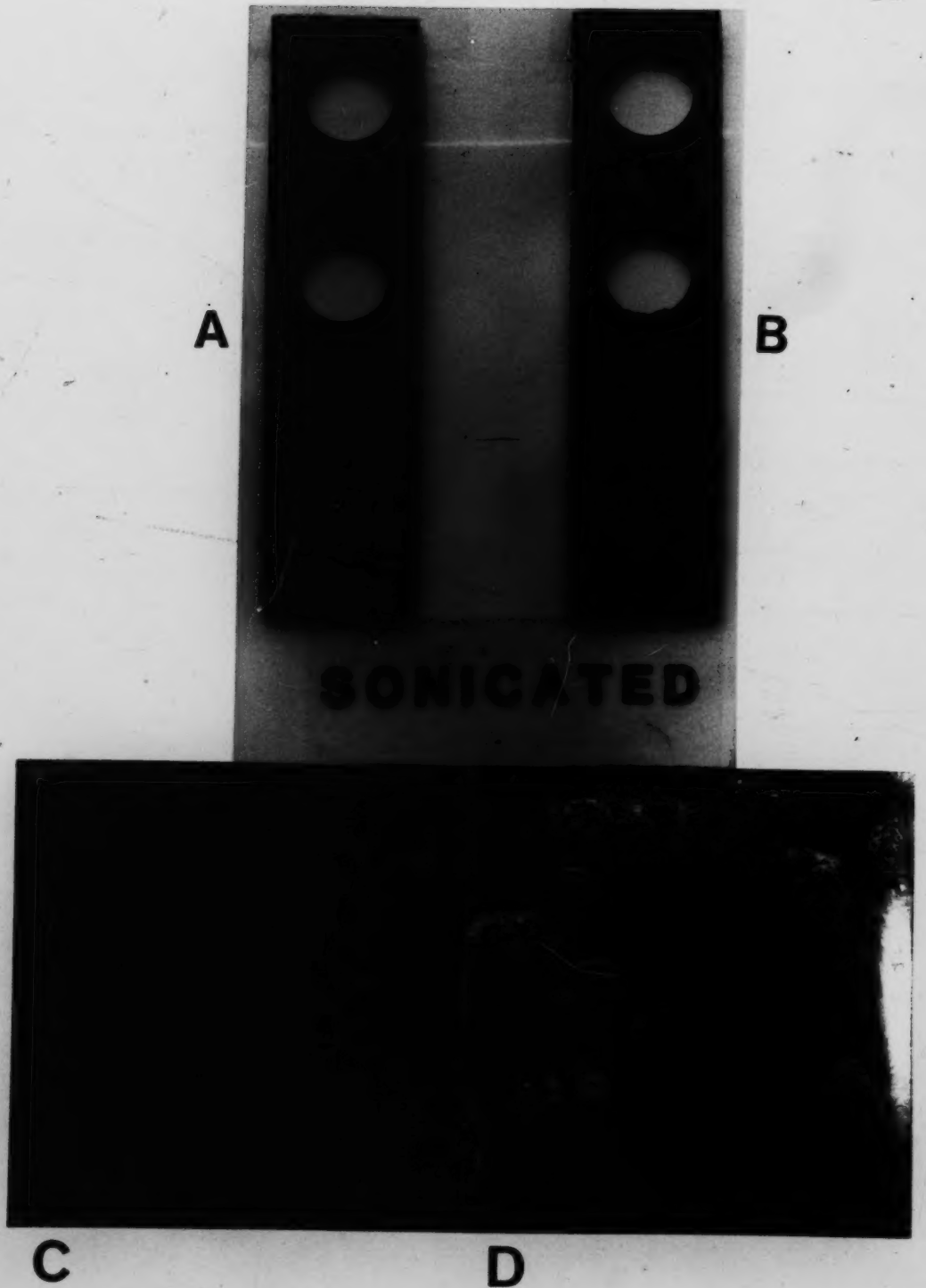
product came off very easily with rinsing, and most of the attached material fell off after fixing and drying. Coupons were covered in a thin, black layer of iron sulfide, similar to that found with isolate Ps 200 +$S_2O_3^=$. Small areas on the coupons with dried, mucoid material remaining were examined and only extracellular mucus-like material with some amorphous FeS was seen. In this case, the organisms attached to the coupoons but did not build up a corrosive FeS layer on the surface. Instead, a passivating FeS film was formed, which resulted in a very low corrosion rate. In this case, in mixed culture, more than just attachment was required for corrosion to occur.

Observations of corrosion under the biofilm

The type of corrosion seen on the mild steel coupons and the weight loss was dependent on the type and amount of adherent material. In tests where sulfide was produced but where there was poor attachment of the organisms and closely adherent, passivating iron sulfide layers were formed (i.e. Ps 200 +$S_2O_3^=$ and mixed in Butlin's), low corrosion rates resulted and no corrosion was observed. On coupons from mixed Butlin's cultures, bright spots, surrounded by a close protective FeS film, were seen after sonication (Figure 25a). These appeared in the areas where the raised patches (Figure 21a) had been removed. These cleaned spots had no difference in topography inside and out (Figure 25c). After 6 weeks incubation, the coupons surface was so rough and the FeS film so thin that these spots could not be distinguished under the SEM. No pitting or corrosion was evident after acid treatment. Similar spots were found on coupons from Ps 200 +$S_2O_3^=$ cultures.

Removal of some of the loosely adherent black product from the heavily coated coupons from AL1-Butlin's (12 week) by sonication revealed shiny, cleaned patches of metal which looked like pits, surrounded by more closely adhering areas of film (Figure

Figure 25. Appearance of 'pre-pits' on mild steel coupons after removal of loosely attached material by sonication. Coupons were exposed for 12 weeks in continuous culture to mixed culture (A) and pure culture of *D. vulgaris* AL1 (B) in Butlin's medium. With mixed culture (C), no topographical difference between the inside and outside of the patches could be seen, and no actual pits could be distinguished. In (B) cleaned patches of bright corroded metal, 0.5-2 mm, were rapidly oxidized and formed rusty orange rings around the patches (^). Other corrosion products in mounds on the coupons were composed of black FeS and some green crystals composed of Fe and P. No pits could be distinguished after acid treatment.


A
B
SONICATED
C
D

25b). The patches ranged in size from 0.5 to 2 mm in diameter and quickly oxidized, turning rusty orange, with a build up of product around the edges as seen in Figure 25d. This remaining material was a combination of rusty oxides and amorphous black FeS, with some greenish crystals of iron phosphate. However, under the SEM, there was no real depression in the metal surface where these "pits" were located (Figure 25d). When the remaining, closely adherent coat was removed by acid treatment, there were also no visible pits or topographical differences in the surface. Coupons were tarnished with a similar patchy appearance of light spots surrounded by darker coloring, suggesting that the patches were not an artifact of sonic treatment but could be 'pre-pits' which may have developed into actual corrosion sites with longer exposure.

Coupons with larger amounts of adhering material, such as AL1 +$S_2O_3^=$, had larger 'chunks' of hard black coating removed, under which were larger, localized patches of bright metal, such as seen on the right hand edge of the Butlin's (12 week) coupon (Figure 25b). After acid treatment, these coupons had the same tarnished patches but no topographical difference between the dark and clean areas was seen under the SEM. Larger patches of corrosion were also found on the Butlin's (6 week) coupons which had more attached material, compared to smaller areas, like pits, on the Butlin's (12 week) coupons which had less biofouling. As expected, the coupons with larger areas of corrosion showed higher weight loss (Table 19). These data reflect the problem of using total weight loss determinations as a measure for pitting or localized corrosion. This does not measure the corrosion rate at the site of corrosion or indicate the true extent of the corrosion problem.

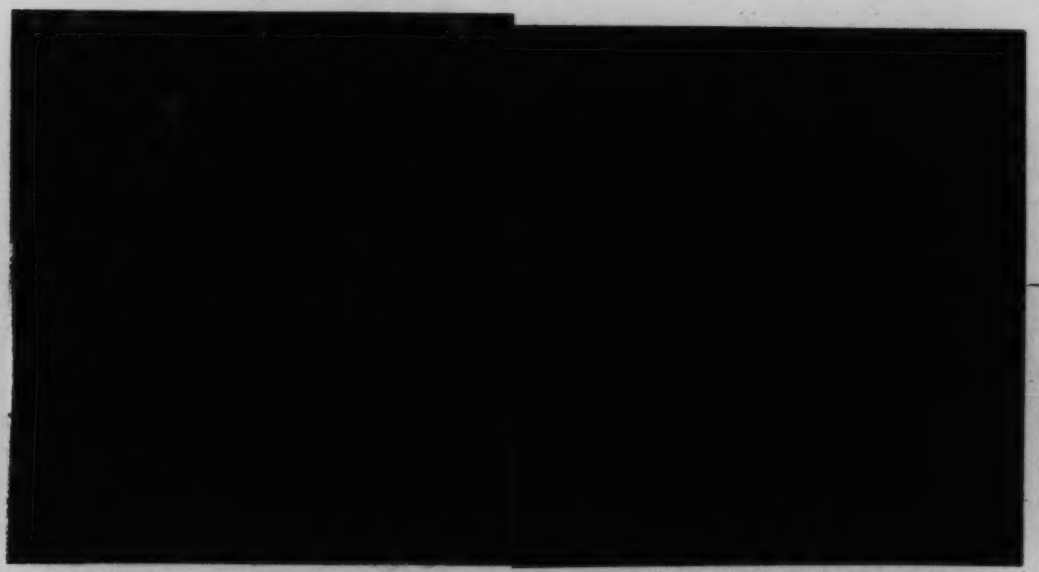
Corrosion tests in Butlin's -YE with *D. vulgaris* AL1 in pure and mixed culture actually showed severe corrosion, which was readily visible without magnification of the coupons (Figure 26,27). On coupons from AL1 -YE, bright metal showed around the washer and during sonication large patches of loosely adherent material were removed,

Figure 26. Appearance of corrosion on the surface of mild steel coupons after 12 weeks exposure in continuous culture to pure culture of *D. vulgaris* AL1 in Butlin's -YE.

A. Acid cleaned coupons showing the corroded patches and localized corrosion and pitting in the area around the washer where biofouling built up. B. Note the corrosion patch with bright, corroded edge. Higher magnification of these areas (D) shows a smoothing and angular appearance and change in crystal structure compared to (C) the surface of a fresh control.

A

B



C D

Figure 27. Appearance of corrosion on the surface of mild steel coupons after 12 weeks exposure in continuous culture to mixed culture of isolates Ps 200 and *D. vulgaris* AL1 in Butlin's -YE.

A. Acid cleaned coupons showed patches of tarnish and bright corroded metal. Note the severe corrosion around the base of the washer.

B. The broad smooth pit at the base of the washer and

C. The corroded surface inside the pit with smoothing and crystals of metal now distinguishable.

A

B

C

showing areas of localized corrosion patches with bright edges. Again, some lumps of iron phosphate and iron sulfides remained on the surface. After acid cleaning, these patches were visible with corroded, rough edges (Figure 26b) as well as localized surface roughening and 'pitting' corrosion, around the bottom of the washer. SEM observation of the corroded bright metal at the border of the patch showed a smoothing of the metal surface and a more angular crystal structure than the rough surface of the control coupons (Figure 26 c,d). The inside of some of the spots on acid cleaned coupons from AL1-Butlin's (6 week) also had a similar angular appearance, which indicated that some corrosion had occurred even in that test.

The most severe corrosion and a higher corrosion rate was found on the most heavily coated coupons from mixed culture -YE. The hard crusty coating was removed in large pieces to reveal large bare patches of shiny corroded metal and sections of closely adhering black coating still visible on the acid cleaned coupons (Figure 27a). Extensive corrosion was seen near the edge of the washer, where the deposits of bacteria and FeS had built up. Examination of the inside of the broad smooth 'pit' (Figure 27 b,c), showed extensive corrosion and smoothing of the surface and changes in the crystal structure where the metal grains are distinguishable. Gaylarde and Johnson (1986) and Mara and Williams (1972) described similar surface changes and intergranular corrosion on coupons exposed to SRB. Crevice corrosion near and under washers, under bacterial deposits, has been described by Tatnall (1986) and Kobrin (1986), which was manifest as a 'gouging' type of corrosion, similar to the corrosion seen here.

General discussion and suggestions for further research

a. Corrosion by biogenic sulfide is related to attachment: The corrosion rates calculated from the weight loss measurements were in the range reported by laboratory studies with pure cultures of SRB (King et al. 1973), but the rates at the areas of severe

localized corrosion would be much higher than the overall rate. Corrosion rates between 1.0 and 4.9 mpy are considered moderate under field conditions (NACE 1975b).

The observations of corrosion by pure and mixed cultures of *D. vulgaris* AL1 are consistent with the expected corrosion results with SRB. In these experiments, attachment, colonization and sulfide production and a build up of FeS film at the surface of the coupons seems to be required for high corrosion rates. The typical thick, slimy black fouling which developed on the corrosion coupons actually was composed of mats of organisms interspersed with amorphous FeS. Gaylarde and Johnson (1986) showed fouling layers consisting of bacteria, and King et al. (1973) mentioned that the black slime had many active bacteria associated with it. As observed by Hardy and Brown (1984) and Mara and Williams (1972), the most intense, localized corrosion under the biogenic sulfide films occurred under loosely adherent areas of the film. Such material was easily removed by sonication, revealing areas of shiny, corroding metal surrounded by tightly bound, more protective material. Cleaned areas varied in number and size from 1 mm spots on coupons which had only thin films to larger localized areas of corrosion on coupons with larger amounts of adhereing material. Further experiments should be carried out for longer time periods to see if the 'pre-pits' under nonadherent film develop into pits, so that pitting measurements could be made.

The general trend in these experiment with *D. vulgaris* AL1 in pure and mixed culture was that the more attachment and corrosion product on the coupons, the greater the corrosion rate. A summary of the approximate amount of attached material, the weight loss due to corrosion of the coupons and a brief description of the corrosion product and biofilm is given in Appendix H. The most heavily fouled coupons (i.e. AL1 $+S_2O_3^=$ and mixed -YE) showed the greatest weight gain as well as higher corrosion rates. Observation of increase in corrosion with increase in biofilm are consistent with the various mechanisms proposed for corrosion by SRB. The organisms attach to and

colonize the surface, producing sulfide and forming a matrix of organisms and FeS which enhances the corrosion of the metal. With thin fouling layers or little attachment, protective FeS layers formed resulting in the passivation of the metal. Even with particulate FeS suspended in the medium, little corrosion was seen because it must be in contact with the metal surface. With thicker fouling layers, the FeS cathode in close proximity to metal surface and the larger FeS cathode provides an increased area for the cathodic reaction (area effect for galvanic corrosion) and increased hydrogen absorption. With an increase in biofilm thickness, there could be an increase in sulfide production (Neilson 1987), the generation of fresh FeS and an increase in the number, and possibly metabolic activity, of the organisms for hydrogenase activity to regenerate the cathode (King and Wakerly 1973). Bacterial action within the film affects the nature and distribution of the film on the surface (Mara and Williams 1972). Sulfide generated from within the biofilm affects the crystal structure of the iron sulfide, causing the breakdown of the protective films and thus the differentiation into corrosive and adherent sections. Herbert and Stott (1983) suggested that the biofilm enhanced the contact between the anode and cathode areas within the film. Clearly, corrosion by SRB and biogenic iron sulfides must be considered in terms of the complexity of the biofilm.

b. Comparison of corrosion by pure and mixed culture in Butlin's -YE: The highest corrosion rate (22.7 ±1.3 mdd or 4.2 mpy) was found with mixed culture in Butlin's medium without yeast extract. *D. vulgaris* AL1 alone also had a high corrosion rate (18.6 ±1.1 mdd or 3.4 mpy) in -YE medium and significant localized corrosion was observed on the coupons only with these tests. It is difficult to compare the two systems, however, because of differences in culture development, which would have affected the corrosion conditions. With mixed culture, biofouling developed on the coupons immediately after inoculation and conditions were likely anaerobic throughout the tests.

However, in the test with pure culture of AL1 incubated for 12 weeks, aerobic corrosion, as in the controls, existed for the first 6 weeks, as the SRB slowly built up fouling. Differential aeration effects may have been active between the areas under the deposits of bacteria and FeS which built up around the washer and in the patches, which could explain the severe corrosion in these areas. Aerated conditions with a biogenic sulfide film are very corrosive conditions (Hardy and Brown 1984). Anaerobic corrosion conditions would have become active in the last 6 weeks, when the coupons were fouled and the cultures had grown and produced sulfide. More replicates and time points would have to be determined to evaluate the differences in the mechanisms of corrosion. Experiments into the effects of aeration on corrosion could be done once the biofilm had developed on the coupons. Additional testing must be done to determine the relative corrosion ability of *D. vulgaris* AL1 in pure and mixed cultures.

c. Effects of media on growth and attachment: There were substantial differences in corrosion rates and the ability of the isolates to colonize the coupons in the different tests and media used in these experiments. Mixed culture studies gave the most varied results. In Butlin's -YE, both organisms grew readily in the medium and attached to the coupons, producing an extensive FeS-rich film which resulted in intense pitting and the highest corrosion rates of the experiment. From the limited corrosion experiments conducted in this study, it appears that attachment and corrosion were enhanced in the medium without yeast extract.

Lower corrosion rates were found in Butlin's medium with yeast extract, which was apparently related to poorer attachment of the organisms and formation of a protective FeS film, even though both isolates grew well suspended in the medium and produced H_2S. The pure culture of isolate Ps 200 grew well suspended in the Butlin's medium but there was little attachment to the coupons. The effect of yeast extract was not as dramatic

with pure culture of AL1 as considerable biofouling and corrosion was observed in the Butlin's-6 week test and in Butlin's with both yeast extract and thiosulfate. However, with Butlin's-12 week test, there was good growth suspended in the medium but less colonization of the coupons and less corrosion was found. In all these cases, the activity and number of organisms on the coupons was independent of the counts and growth in the medium.

The 'inhibition' of corrosion found with yeast extract seems to be associated with poorer attachment of the organisms to the coupons. Gaylarde and Johnson (1986) reported large variations in corrosion rates in mixed and pure culture experiments in Postgate B medium, which contains yeast extract. Could the low corrosion rates they reported in triple mixed cultures of *D. vulgaris*, *V. anguilarum* and a *Citrobacter* sp. have been related to differences in the ability to attach?

Further corrosion experiments should be carried out in medium without yeast extract or in actual produced water. Investigation into the nutritional conditions which increase the attachment and corrosion ability of SRB, isolate Ps 200, and mixed cultures should be studied to evaluate if increased attachment was the reason why medium -YE results in higher corrosion rates. It will be important to define the nutrient limitations present in the oil field environment, and determine the minimum requirements for growth and activity of *A. putrefaciens* strains and SRB. What effect do various nutrient limitations such as iron, phophorous or nitrogen have on the characteristics important to corrosion such as exopolysaccharide production, attachment ability or reductive activities (Brown and Williams 1985; M.R.W. Brown, personal communication)? Factors such as carbon and nitrogen limitation, growth phase and varying carbons sources have been found to modify bacterial attachment and activity at surfaces (Brown et al. 1977; Wardell et al. 1983; Sutherland 1983; Fletcher 1977, 1985). The effects of growth rate as well as nutrient limitation on attachment could be tested using a chemostat with the overflow to a

flow-through Robbins device (McCoy et al. 1981).

d. Corrosion by *A. putrefaciens* isolate Ps 200: Corrosion by isolate Ps 200 under iron-reducing and sulfide-generating conditions in these experiments was inconclusive because the organisms showed poor attachment to the coupons. The corrosion tests with this isolate in Butlin's medium were set up to determine the effects of iron reduction in corrosion but the effects of removal of passive film were not clearly demonstrated in these experiments. Attachment to the surface and direct contact with the Fe_2O_3 would still be required for iron reduction to occur. If the passive film was removed from the coupons, it appeared that iron reduction alone was not a major mechanism for anaerobic corrosion under the noncorrosive, anaerobic conditions created by growth of the organisms. The effects of passive film removal and the role of iron reduction in corrosion could be tested by following the removal of passive film after oxidation of the coupons had occurred, such as in the control, after inoculation with isolate Ps 200. Under alternating aerobic and anaerobic conditions, as passive iron oxide films were alternately formed and removed, the action of Ps 200 was more clearly evident (C.O. Obuekwe, Ph.D. thesis). The role of this isolate in corrosion could also be tested using actively aerated conditions, and compared to an aerated control. Corrosion would likely be enhanced by *A. putrefaciens* strains if they formed an uneven biofilm on the coupons and caused differential removal of the passive film (depassivation) under the deposits which creates differential concentration cells, especially under aerated conditions (Hamilton and Maxwell 1986).

The relative importance of iron reduction and sulfide reduction in the corrosion activity of isolate Ps 200 could not be evaluated in these experiments. Experiments could be set up to test for the passive film removal-under sulfide generating conditions (i.e. oxidized control + Ps 200 +$S_2O_3^{=}$) but it will still be difficult to determine whether iron

reduction mechanisms are active under sulfide-generating conditions, or in mixed culture situations with SRB, since both activities result in disruption of the ferric oxide passive film (C.O. Obuekwe, Ph.D. thesis; Vidella 1986).

Generation of sulfide by isolate Ps 200 in pure culture created a protective FeS film, probably as a result of poor attachment. Low corrosion rates (0.25 mpy) were also seen under sulfide generating conditions by Obuekwe (Ph.D. thesis), but some pitting was evident. Similar low corrosion rates were reported by Gaylarde and Johnson (1986) with *Citrobacter* sp. which produced sulfide due to putrefaction and a protective FeS film, leading them to conclude that H_2S was not important to corrosion. However, results of the present experiments with mixed and pure cultures of SRB indicate that sulfide production, combined with bacterial attachment and extensive fouling of the coupons to form an unprotective iron sulfide layer, is needed to enhance corrosion.

Further investigations into corrosion by Ps 200 and related isolates under sulfide generating conditions should be carried out under conditions which support attachment, for example, in media without yeast extract. Comparison of the corrosive activity and type of sulfide film and biofouling produced by sulfite-reducing bacteria (eg. *A. putrefaciens* strains) and SRB would be interesting to evaluate the relative affect of the activity of the organisms vs FeS formation on corrosion. *A. putrefaciens* strains should be also be tested for the presence of hydrogenase enzymes before comparison can be made to the SRB. The factors affecting the formation and differentiation of sulfide films could be further investigated with SRB, *A. putrefaciens* strains and mixed cultures.

c. Vivianite formation in corrosion products: Crystals of iron phosphate formed on coupons incubated with isolate Ps 200 in Butlin's -YE. Iron phosphates were also found in the corrosion products on the coupons in pure cultures of *D. vulgaris* AL1. A few similar crystals were also visible on coupons incubated with isolate Ps 200 in

Butlin's medium in these tests and by Obuekwe et al. (1981b). Other researchers often have found iron phosphate in the corrosion products from corrosion sites in the field (Booth et al. 1962; Worthingham et al. 1986) and in the lab (Iverson 1981; Pankhania et al. 1986b). Another common corrosion product, iron carbonate (siderite) (Worthingham et al. 1986), is also produced during growth of iron-reducing cultures in low phosphate media (C. Panter, M.Sc. thesis).

Iverson and Olson (1983; Iverson et al. 1986) have used the observation of vivianite formation during corrosion as partial support for a corrosion mechanism involving a corrosive phosphorus compound. However, it appears that iron phosphates form readily in phosphate-containing medium when ferrous ions are produced, both during iron reduction and corrosion. The phosphorus metabolism of *A. putrefaciens* strains could be studied to see if there is a precedent for phosphate reduction by these organisms, as has been suggested for *Desulfovibrio* (Iverson et al. 1986). Do these bacteria have an active or passive role in the formation of iron phosphates? Vivianite formation could be tested in media with lower concentrations of phosphate, or in produced water to see if it forms under field conditions with isolate Ps 200. This would also allow for the evaluation of corrosion by *A. putrefaciens* strains under iron-reducing conditions without this interfering precipitate.

f. Corrosion tests with pure cultures of *D. vulgaris* AL1: These experiments highlight the problem of getting reproducible results with pure cultures of SRB under experimental conditions where the media are not poised and anaerobiosis is not imposed. Similar problems were also noted by Fedorak et al. (1987) when determining the MPN of pure cultures of SRB using unsealed tubes. Interestingly, the SRB established growth and caused corrosion in pure culture even without anaerobic conditions being imposed at the time of inoculation. Growth of anaerobe *D. vulgaris* AL1 in pure culture was highly

variable, with large variation between tests with pure culture, even in the same medium. Highly variable weight loss results were also reported in low iron media by King et al. (1973) and by Gaylarde and Johnson (1986). This is not unexpected, considering the type of corrosion attack which depends on the number of sites developed for localized or pitting corrosion. In addition, cultures in non-ideal, nutrient limited media (-YE), which are more like environmental conditions, take a long time to establish. Such conditions are perhaps more representative of the 'real world', but are hard to reproduce experimentally. Sulfate-reducing bacteria show varying oxygen tolerance (Cypionka et al. 1985) and are very persistent, which explains why they survive and establish in anaerobic niches even if the surroundings are aerobic.

g. Interaction in mixed culture: Growth in mixed culture with isolate Ps 200 affected the ability of the SRB to grow suspended in the medium, especially in medium -YE, and greatly affected the tendency for the organisms to attachment to the coupons. Although the highest corrosion rates were seen with mixed culture, it could not be determined whether isolate Ps 200 helped increase corrosion by simply reducing the environment or by taking an active part, possibly by increasing sulfide production through synergistic activity with the SRB. Corrosion tests could be done in mixed culture with other aerobic, non-corroding bacteria found in the oil field, to test whether similar effects on attachment and corrosion are seen. Tests with other aerobic organisms could also provide an 'anaerobic' control for the corrosion tests.

The nature of the interaction between SRB and sulfide-generating *A. putrefaciens* strains could be studied using radiorespirometry techniques, such as those developed by Rosser and Hamilton (1983) which measure the turnover of ^{35}S-lablled sulfate. This could determine the differences in metabolic activity, utilization of various carbon sources and sulfide-generating activity between mixed and pure cultures. Monitoring the sulfide

production or carbon utilization of the organisms attached to coupons could help determine changes in surface activity which affect corrosion and investigate interactions within the biofilm (Hamilton 1985). This could also determine if the increased fouling correlates with increased activity in mixed culture or increased sulfide production, although this is not always the case in natural situations (Sanders and Maxwell 1983).

h. Differentiation of organisms: *D. vulgaris* AL1 attaches well on its own and in mixed culture but observation in the SEM could not readily differentiate between the two organisms, which both form long, filamentous rods. The black, slimy corrosion films were primarily bacteria interspersed with FeS and SRB were not only in the lower reaches but throughout. Methods to differentiate and localize iron-reducing Ps 200 in a biofilm, such as cell surface fluorescent antibody probes, should be constructed (Zambon et al. 1984). Such probes would be useful for identifying and localizing *A. putrefaciens* strains and SRB in actual microbial corrosion sites and within biofilms in the oil field. Much of the recent corrosion work has involved the development of diagnostic tests for the detection of corrosion bacteria (Pope 1986). Another means of identifying organisms in microbial communities is to look for 'signature' compounds (White 1983; White et al. 1979). Some work has been done on determining the fatty acid and phospholipid profiles of a limited number of strains of *A. putrefaciens* (Wilkinson and Cauldwell 1980) and isolates, including Ps 200, have been submitted for analysis to Dr. D.C. White for evaluation. Results should be useful for comparison to previous work and to determine if there are any unique compounds useful for identification of these bacteria in the environment.

Observation of the pitting and material attached in an actual pipeline and comparison to the black slime that developed on the corrosion coupons in the lab would indicate whether extensive bacterial biofilms occur in oil field situations. Chemical (i.e. protein,

FeS type and concentration) and cell number analysis of the biofouling attached to coupons could be done to find the proportion of cells to FeS and compare that to results of field analysis, such as determined by Sanders and Hamilton (1986). It is important to determine if the corrosion observed and rates measured in the lab experiments can be related to actual corrosion in the field.

i. Summary: The results of these laboratory corrosion experiments showed that the greatest corrosion of mild steel was found with mixed culture and in nutrient-limited medium, both conditions which would be found in the oil field situation. Growth in mixed culture of two commonly-occurring oil field bacteria, *A. putrefaciens* and *D. vulgaris*, resulted in better attachment and corrosion than pure cultures in medium without yeast extract. In all media tested, both attachment and sulfide production were required for high corrosion rates. Although these corrosion rates calculated were lower than reported for field observations, the severe localized corrosion, such as that observed on test coupons, could eventually result in corrosion failure in a pipeline.

V CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH

Iron-reducing, sulfide-generating *Alteromonas putrefaciens* have been readily isolated from saline produced water from oil fields in Alberta. They are an important component of the mixed microbial community associated with the oil field environment and have been implicated in the corrosion of oil pipelines. These bacteria are widely distributed and comprised a substantial portion of the aerobic microbial population in some oil fields. A total of 80 isolates, similar to Obuekwe's isolate Ps 200, were isolated from five different oil fields and have the characteristics of *A. putrefaciens* described in the literature. The ability to reduce ferric iron and participate in sulfide generation by reduction of thiosulfate and/or sulfite were additional characteristics which help to identify and differentiate strains of this organism from *Pseudomonas* and *Alteromonas*. Strains with characteristics representative of the four DNA homology groupings of Owen et al. (1978), were isolated and differentiated based in part on salt tolerance, the ability to grow at 4°C, and G+C content. An additional differential characteristic was proposed for isolates in Groups 2 and 3, based on the ability of isolates to reduce thiosulfate but not sulfite. Only a limited number of these strains were available for comparison so more isolates which reduce only thiosulfite should be tested to confirm this observation.

The subclusters of *A. putrefaciens* strains have similar characteristics and all could potentially contibute to corrosion in the field. Differentiation of groups is of interest, however, in determining not only how such differences might affect their distribution but also their activity in the environment with respect to attachment, iron reduction, sulfide generation, interaction with SRB and corrosion. Groups of *A. putrefaciens* were differentiated based on salt tolerance and various strains should be tested for a Na^+ requirement for growth in chemically defined medium. Stenberg et al. (1984) found that sodium was required for amino acid transport during TMAO reduction using some amino acids. Such a sodium requirement should also be tested for iron and sulfite reduction.

Fluorescent antibody probes (Zambon et al. 1984; Pope 1986) or genetic probes could be useful for identifying and localizing *A. putrefaciens* strains within biofilms or in microbial corrosion sites in the field. Such probes could be used to determine the cross reactivity between the four groups and determine their relatedness to other organisms such as *Pseudomonas* and *Alteromonas* strains. Further work on the differentiation of these bacteria could include a determination of fatty acid profiles (Wilkinson and Caudwell 1980) and comparision of the 5s rRNA sequences of the four groups to help determine their relationships to the *Alteromonas* genus and the placement in a separate genus as proposed by MacDonnell and Colwell (1985). Characterization of this organism is an interesting example of the developments in bacterial taxonomy.

Physiological differences between these isolates make them very adaptable to the oil field environment, as some grow at low temperatures and others could tolerate a highly saline oil field environment. There was a slight difference in the distribution of salt tolerant strains, perhaps due to the selective conditions of high salinity. Identification of these as strains of the genus *Alteromonas*, which is predominantly from the marine environment, is of interest. What are the origins of these organisms in the oil field fluids? Are they native to the formation or introduced through production activity? They are prevalent in the lower salinity samples, and in fluids from fields using waterflooding techniques for oil recovery. These activities could be introducing the organisms or may be diluting the salinity of the ground water and increasing the nutrient load (i.e. sulfate), which could promote the growth of the organisms (Moses and Springham 1982; King and Stott 1983). Further sampling is needed to test the distribution of bacteria in various sources of injection water, such as lake water and in water injection systems, to determine their source in the oil field systems. Producing oil fields that showed high levels of iron-reducing bacteria, such as Cold Lake (which uses steam injection techniques for the recovery of heavy oil), should be surveyed to test the distribution of these organisms throughout the system. Based on the heavy load of bacteria and high water content in the

heavy oil, high corrosion rates could be predicted for this system. It would be interesting to investigate this field for potential microbial corrosion problems, and also correlate their presence and activity with actual corrosion damage. Do these bacteria, including *A. putrefaciens* and SRB, occur in actual corrosion sites in a pipeline?

Strains of*A. putrefaciens* are important environmental isolates as they are active in anaerobic cycles and have economic impact because of their ability to reduce a wide range of terminal electron acceptors including NO_3^-, TMAO, Fe^{3+} and $SO_3^=$. The sulfide-generation and TMAO-reduction characteristic is important to fish spoilage whereas iron reduction and sulfide generation have been implicated in corrosion. All isolates tested reduced ferric iron at about the same rate per unit protein in a soluble ferric iron assay and representative stains reduced insoluble Fe_2O_3, but at lower rates. Consequently, all isolates should be able to enhance corrosion by solubilizing ferric oxide passive film. This species had not previously been reported to reduce ferric iron and this ability has not been studied except by Obuekwe (Ph.D. thesis) and Arnold et al. (1986a; 1986b). The presence and possible role of these organisms in other environments where iron and sulfur cycling are taking place, such as marine sediments and soils, should be investigated. The level of iron-reducing activity, and the nature of the iron reduction enzyme systems of *A. putrefaciens,* should be compared to other iron-reducers isolated such as the coryneforms, Enterobacteriaceae, clostridia and *Bacillus* spp. The rates of iron reduction on insoluble oxides also should be compared under similar conditions to confirm that *A. putrefaciens* strains have high iron-reducing activity. The corrosion activity of other iron-reducing bacteria found in the oil field could be tested, especially those that do not produce sulfide, to compare the relative importance of both mechanisms in corrosion.

A. putrefaciens has been previously characterized by its ability to produce sulfide from thiosulfate in complex media. They in fact were found to have an active sulfite

reductase system, based on their ability to grow anaerobically on sulfite, producing sulfide, and based on the inverse isotope fractionation pattern produced. Further enzymatic characterization of sulfite reductase and comparison to that produced by sulfite-reducing clostridia and SRB would confirm the nature of the dissimilatory reduction pathway in this organism. The differences in enzyme systems in the organisms that only reduce thiosulfate could be determined. This organism would be a good candidate to study the mechanisms for the inverse isotope effect, because it is strictly oxidative and will not grow anaerobically without an electron acceptor such as sulfite. Optimizing the production of ^{34}S-enriched sulfide by being able to accurately predict the location of the inverse isotope maxima could be a valuble method to produce high $\delta\,^{34}S$ label which could be used in agriculture and research (Dr. H.R. Krouse, University of Calgary, personal communication).

The conditions for induction and the interaction between the various reductase systems should be studied to determine which systems are active under what conditions. This is required to determine the conditions under which sulfite reductase and/or iron reductase would be active in a corrosion situation, or in the environment. The characteristics of the various reductase systems have been studied independently by a series of workers before the combination of electron acceptors which these organisms can use was known. Nitrate and TMAO reductases are inducible when the substrate is present and under microaerophilic conditions (Easter et al. 1983) and a second anaerobically-induced iron reductase system was described by Arnold et al. (1986a). The iron reductase system characterized by Obuekwe et al. (1982a) was found to be constitutive in iron-containing media. The inhibitory effects of nitrate on iron reduction (Obuekwe et al. 1981d,1982b) and TMAO reduction (Easter et al. 1983) have been studied separately. Further investigations are necessary to determine the substrates used by each reductase system and whether TMAO or nitrate reductase have some iron reduction activity. The induction, interaction and characteristics of the two iron reductase

systems should be further investigated, as well as the effects on the iron reductase assay. The various reductase systems could be characterized and compared to those found in other strains of bacteria.

A. putrefaciens is an important oil field bacterium to study because it can enhance corrosion on its own (C.O. Obuekwe, Ph.D thesis) and greatly affects the corrosion-abilities of SRB. The corrosion-enhancing ability of isolate Ps 200 was not clearly demonstrated in the experiments described in this thesis. They did not attach well to the coupons under iron-reducing or sulfide-generating conditions and growth of the bacteria resulted in non-corrosive anaerobic conditions. More work should be done to define the conditions which demonstrate corrosion by islolate Ps 200 such as alternating aerobic and anaerobic cycles (C.O. Obuekwe, Ph.D. thesis), aerated conditions or removal of passive film from a corroded surface. Such conditions could be found in water handling systems, or in tanks where the water level fluctuates.

Growth and attachment of isolate Ps 200 were affected by the composition of the medium and further investigations should be done to define the conditions that promote attachment to metal surfaces. The changes in cell surface that occur during growth and in various media could have an effect on attachment and should be investigated. Techniques could involve using cell surface hydrophobicity measurements (Rosenberg et al. 1980; Rosenberg 1981), determination of contact angles as well as measurements of attachment to various surfaces (Dexter et al. 1975; Brown et al. 1977, Pringle and Fletcher 1983). The production of glycocalyx by a related organism, *P. atlantica*, has been studied by Uhlinger and White (1983) and the effects of this exopolymer on corrosion have been investigated (Nivens et al. 1986). The factors which affect exopolysaccharide production by *A. putrefaciens* strains should also be studied as well as the ability of the exopolymers to concentrate metal ions (i.e. Fe^{3+}, Cl^{-} and Mn^{3+}) (Mittelman and Geesey 1986). The range of metals reduced (i.e. Cr, Cu, Ni, Pb, Sn and Mo) (Jones et al. 1984a) by *A. putrefaciens* strains, in addition to iron and manganese (Obuekwe et al. 1982b), should be

tested. This organism could possibly disrupt the passive films and enhance corrosion of other industrial metals. Attachment to and corrosion of other metals and alloys such as stainless steels, copper, aluminum, nickel or titanium could be tested in the future.

Attachment, biofilm formation and the build up of iron sulfides on the surface of the metal coupons was required for corrosion to occur in pure culture *Desulfovibrio vulgaris* and mixed culture with *A. putrefaciens*. The corrosion rate and amount of corrosion appeared to increase with an increased amount of fouling on the coupons, consistent with the various mechanisms of corrosion proposed for SRB. Corrosion occurred in localized areas under loosely adherent, non-protective sections of the biofilm, as noted by Hardy and Brown (1984) and Mara and Williams (1972). The heavy black, 'slimy' corrosion film which coated the mild steel corrosion coupons was a complex mixture of heavy mats of bacteria interspersed with FeS. Clearly, any investigation into the mechanisms of biological corrosion must consider the complex activity of the biofilm where many processes are active. The microorganisms affect the formation, distribution, differentiation and corrosion activity of the film. The recommended practice for effective control of microbial corrosion problems is prevention of a buildup of biofouling on the metal surface by regular scraping, pigging of pipelines and preventing stagnant areas in the system (NACE 1975a)

In corrosion tests in mixed culture, isolate Ps 200 greatly influenced the attachment of *D. vulgaris* AL1 to the coupons in different media used and thus the corrosion rate and the extent of corrosion that was observed. In the media with yeast extract, poor attachment and low corrosion rates were seen whereas the greatest amount of attachment and corrosion was found in mixed culture in medium without yeast extract. In these corrosion tests, the high bacterial counts and good growth of both organisms in the medium could not be used to predicted the activity and number of organisms on the surface of the coupons. The corrosion test results demonstrate a problem found in oil field sampling; high numbers of planktonic organisms do not always correlate with high

numbers of attached bacteria and in contrast, there could be lots of attachment while the numbers of bacteria suspended are very low. The nutrient conditions and composition of the bulk fluid greatly affected attachment of organisms to the coupons. Investigation into the nutrient conditions in oil fields and defining the conditions which promote attachment of both *A. putrefaciens* and SRB will be important to be able to predict when they are most active.

Although the highest corrosion rates and most severe localized corrosion were found in mixed culture, the nature of the interaction between *A. putrefaciens* and SRB requires further study. Is there an exchange of sulfate reduction intermediates and does sulfite and thiosufate reduction by isolate Ps 200 increase the sulfide production in mixed culture? What are the sources and concentrations of oxidized sulfur intermediates in the oil field environment? If sulfate concentrations are limiting, the use of bisulfite as an oxygen scavenger for inhibition of corrosion could possibly enhance the sulfide production in the system. The corrosion activity of other sulfite-reducing organisms, such as the clostridia, which are found in the oil field and are likely interact with SRB and *A. putrefaciens* could also be tested in mixed culture.

From the limited corrosion experiments conducted in this study, it appears that attachment and corrosion were enhanced using conditions recommended by Tatnall (1986), which mimic field conditions. These include using nutrient limited media (without yeast extract) and mixed cultures. The corrosive effects of aerated conditions were demonstrated by a pure culture of *D. vulgaris* AL1 in -YE, where the fouling developed over a long time period. One can envision even more complex, nonhomogeneous corrosion conditions in the field including mixed culture with other organisms, larger amounts of iron sulfide fouling, long lengths of pipe to act as a cathode and possibly aeration. Laboratory corrosion studies cannot mimic the total situation, and it is important to analyse corrosion sites in the field to confirm the observations in the lab. Being able to duplicate the higher corrosion rates found in field is not necessary, but it is

important to be able to define the organisms involved in the field, determine the conditions when they are active (pH, temperture etc.) and define the corrosive conditions and chemicals they produce to be able to combat corrosion problems, and design against them (Stoeker 1986).

The process of microbially enhanced corrosion is very complex. The activity of mixed cultures, the effects of media composition on attachment and the effects of aeration on corrosion warrant further study to help define corrosion conditions as they occur in the oil field environment. Organisms such as *A. putrefaciens*, are an important component of the microbial population in the system which should be included in any further study of microbial corrosion.

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VII APPENDIXES

APPENDIX A

MEDIA FORMULATION AND METHODS

1. Modified Butlin's Medium (A.M. Jobson, Ph.D. thesis)

	g/l
K_2HPO_4	0.5
NH_4Cl	1.0
Na_2SO_4	2.0
$MgSO_4 \cdot 7H_2O$	0.1
$Fe_2SO_4 \cdot H_2O$	0.01
Sodium Lactate (60%)	1.5 ml
Yeast Extract	1.5
pH 7.2	

2. B10 Medium (C. Panter, M.Sc. thesis; C.O. Obuekwe, Ph.D. thesis)

	g/l
K_2HPO_4	0.8
KH_2PO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.2
$MnSO_4$	} 1.0 ml of a
$NaMoO_4$	} combined 0.1% solution
$CaSO_4$ (saturated)	10 ml
Yeast Extract (Difco)	5.0
Bacto Peptone (Difco)	5.0
Soluble ferric phosphate ($FePO_4$) (City Chemical Corp., New York)	4.7
dH_2O	990 ml
pH 7.2	

Dissolve $FePO_4$ before adjusting pH of the medium. The color after pH adjustment should be gold to brown. For agar plates, add 20 g/l agar. For sloppy agar tubes, use 2.5 g/l agar and dispense after autoclaving into sterile tubes.

3. Mayfield and Innis (1977) Flagella Stain

Stock Solutions	Proportions
Saturated $KAl(SO_4)_2$	5
20% Tannic acid	5
Saturated $HgCl_2$	2
Saturated basic fuchsin (alcoholic)	0.8

Filter solution through a millipore filter before use.

Modifications to the procedure:

More concentrated, old solutions work best.

1. Add a small drop of 24 h broth culture to a microscope slide.
2. Next to this add a drop of staining solution.
3. Cover both drops with a coverslip. Check along converging line between the drops for stained flagella after 1 min.

Proteus vulgaris (24 h broth culture), which has peritrichous flagella, was used to test the staining method.

4. Glucose or Lactose Oxidative/Fermentative (O/F) medium (Board and Holding 1960)

Solution A	g	Solution B
$NH_4H_2PO_4$	0.5	Glucose or Lactose 10g/200 ml
K_2HPO_4	0.5	
Yeast extract	1.0	Autoclave separately.
Bromothymol blue	0.030	
Agar	15.0	
dH_2O	800 ml	

Adjust to pH 7.15 - green.

Color change on agar plates: yellow = acid, blue = alkaline

Test organism: *P. aeruginosa* -> acid reaction

5. Synthetic (defined) medium for sulfite or thiosulfate reduction (Obuekwe et al. 1983)

	g/l			
K_2HPO_4	0.5			
NH_4Cl	1.0			
$CaCl_2.H_2O$	0.15			
Na_2SO_4	2.0	or	NaCl	1.5
$MgSO_4 \cdot 7H_2O$	0.1	or	$MgCl_2$	0.1
$Fe_2SO_4 \cdot H_2O$	0.01	or	$FeCl_2.4H_2O$	0.07
Sodium Lactate(60%) (Fisher)	1.5-3 ml			
dH_2O	1 l			
pH 7.2				

Chloride constituents can be substituted for sulfur compounds if testing for $SO_3^=$ or $S_2O_3^=$ as the sole sulfur source. Add Na_2SO_3 or $Na_2S_2O_3$ as filter sterilized 10% solutions at a rate of 3 drops (~0.1 ml) per 10 ml of medium.

6. Ornithine decarboxylase

Decarboxylase Base (Difco)

	g/l
Bacto Peptone	5
Yeast extract	3
Dextrose	1
Bromocresol purple (1.6%)	0.625 ml
Cresol red (0.2%)	2.5 ml
L-ornithine (Calbiochem)	10
pH 6.5	

1. Set up duplicate tubes of medium with and without added ornithine.
2. Inoculate test and control tubes. Overlay with 10mm of sterile mineral oil.
3. Observe the color change for 7 days. A positive reaction is a violet to red violet color in the test + ornithine tubes.

7. Nitrate reduction test

Nitrate reduction medium (Stanier et al. 1966)

	g/l	
Yeast extract	3.0	
Glycerol	10.0	
KNO_3	10.0	
Agar	1	(Make overlay with 10 g/l)

1. Dispense medium into tubes after sterilization.
2. Inoculate molten medium, cool in ice then overlay with 5 ml of the same medium.
3. Incubate tubes for up to 1 week, watching for gas production (N_2).
4. Test for nitrite formation with 3 drops each of:
   8 g/l sulfanilic acid in 5 N acetic acid
   5 g/l alpha naphthylamine in 5 N acetic acid

Inject into the tube with a pasteur pipet. Color change to red indicates the presence of nitrite.

Control organisms: *P. aeruginosa* -> gas production
P. vulgaris -> nitrite production

8. Reduction of Trimethylamine oxide (TMAO)

TMAO Medium (Wood and Baird 1943)

	g/l
Bacto peptone	5
D-glucose	2.5
K_2HPO_4	1
$MgSO_4 \cdot 7H_2O$	1
NaCl	5
Trimethylamine-N-oxide	1
pH 7.2	

Dispense 5ml per each 16X150 mm test tube. Inoculate and incubate at 22°C for 2-3 days.

8. Reduction of Trimethylamine oxide (TMAO) (Continued)

Detection of trimethylamine (TMA) production:

1. Add 1.5 ml of 40% formaldehyde. Shake and let tube sit for 3 min.
2. Add 3 ml saturated K_2CO_3.
3. Close tube with Knotes rubber stopper and a plastic cup containing a filter paper strip impregnated with Bromothymol Blue indicator (0.05% in 0.1 N H_2SO_4, pH 4.0)
4. Incubate tubes in water bath at 45°C for 30 min. TMA will distill into the paper, shifting the indicator pH to alkaline (blue) (see photo A).

The Laycock and Reiger method of using indicator-soaked filter paper inverted over the tubes (photo B) gave confirming results but may give false positives if carbonate is on the rim of the tube and does not provide a good seal so that it was sometimes difficult to see which tubes gave the positive reaction.

P. vulgaris and ATCC strains of *A. putrefaciens* were used as positive controls and *P. aeruginosa* as a negative control organisms.

A B

9. B+N mineral medium for oil degradation

	g/l
K_2HPO_4	0.5
Na_2SO_4	2.0
$MgSO_4 \cdot 7H_2O$	0.2
NH_4Cl	1.0
KNO_3	2.0
$FeSO_4.7H_2O$	trace

10. Sulfite reduction /Sulfur isotope fractionation medium

	g/l
K_2HPO_4	0.5
NH_4Cl	1.0
NaCl	1.5
$MgCl_2$	0.1
Sodium Lactate (60%)	1.5 ml
Yeast Extract	1.5
pH 7.2	

APPENDIX B

Calculation of mol % G+C from melting temperature (Tm)

Determination of the melting temperature (Tm) was done as described by Johnson (1981).

DNA preparations were dialysed into 1/2 SSC* overnight. Each dialysis batch must include *E.coli* b DNA as a standard. Melting profile determination was done on a Pye Unicam PU 8800 recording spectrophotometer with an Accuron spx 876 series 2 Temperature Programme Controller supplied by Dr. J. Robinson in the Dept. of Medical Microbiology, University of Alberta.

1. DNA samples were diluted into 1/2 SSC* to Absorbance at 260nm of about 0.4 - 0.45.
   Set machine offset = 0.4
   Full scale span = 0.2 units (A_{260} raises about 0.15 Absorbance units)
2. The cuvette chamber was heated, increasing the temperature linearly at 1°C/min.
3. The melting curve of increasing A_{260} with increasing temperature was recorded on the chart recorder. The temperature was marked on the trace every degree since the temperature increase was not linear at high temperatures with the instrument used.
4. Replot the data of A_{260} vs. temperature and determine the midpoint temperature. The Tm was also confirmed from the recorded trace.
5. Calculate the G+C by comparing the melting temperature of the sample DNA to the same batch *E. coli* b DNA by the formula:

$$\text{mol\% G+C} = \text{mol \% G+C}_{ref} + 1.99\,(Tm_{(x)} - Tm_{(ref)})$$
$$= 51.0 + 1.99\,(Tm_{(x)} - Tm_{(ref)})$$

*SSC is Standard Saline Citrate (0.15 M NaCl + 0.015 M Sodium Citrate, pH 7.2)

APPENDIX B (continued)

Table B1. Determination of mol% G+C of *A. putrefaciens* isolates by the thermal melting point method

Isolate	Melting temperature (Tm)	Calculated mol% G+C	
Batch 1			
E.coli b	87.6 (Tm ref)	51.0 (ref)	
Ps 200	83.2	42.4	
T2Pem 12	83.5	42.8	
ESSO 4-1	84.0	43.8	
ESSO 1-1	84.5	44.8	
T2Fed 3	86.8	49.4	
CL41	87.8	51.4	
Batch 2			
E. coli b	87.6 ± 1.2	51.0 (ref)	
213	84.2	44.2	
216	84.8	45.4	
CL 70	86.4	48.6	
Fed 80	88.0	51.8	
AN24 (Red)	87.7	51.2	
P. aeruginosa ATCC 10145	95.8	67.3	
Batch 3			
E. coli b	86.5 ± 0.1 [a.]	51 (ref) (Tm ref = 86.5)	(Tm ref = 87.6)
CL31	88.1	54.2	52.0
ESSO 1-3	86.4	50.8	48.5
Fed75	88.8	55.6	53.3
AN22 (Fed)	88.6 ± 0.8	55.2 ± 1.5	52.8 ± 1.5
CL71	89.0 ± 0.6	56.0 ± 1.3	53.6 ± 1.3
A. putrefaciens ATCC 8071	84.6 ± 1.6	47.2 ± 3.1 [b.]	44.9 ± 3.1

[a.] Tm ref of *E. coli* for Batch 3 was lower than the previous two determinations. The mol% G+C values calculated with both Tm values are shown. However, the G+C determinations using the lower control value have been reported, because they were dialysed in the same buffer.

[b.] The mol% G+C values calculated may be high by about 2.4 mol% as the values reported for ATCC 8071 were 44.7 (Lee et al. 1977) and 43.4 (Owen et al. 1978).

APPENDIX C

Lowry protein assay for resting cells (Lowry et al. 1951)

1 mL of cell suspension is added to 1mL 1N NaOH in triplicate for alkaline hydrolysis. Tubes are incubated till cloudiness disappears (~ 1 h). The volume was made up to 4 mL with dH_2O. Assay 0.1 to 0.4 ml of this mixture for determination of total protein.

Method: Make up fresh reagent for each assay:

Solution A: (Good for 8 hours)

	Ratio
2% Na_2CO_3 in 0.1N NaOH	100
2% sodium tartrate	1
1% $CuSO_4 \cdot 5H_2O$	1

Solution B: Folin-Chilcalteau reagent (Phenol Reagent: Fisher)
Dilute 1/2 with water.

Standard curve for protein concentration was made with bovine gamma globulin standard (1.4 mg/ml) diluted to from 0-120 ug/ml.

1. Add a total of 0.4 ml protein solution to a small test tube. If smaller volumes are used, make up the difference with dH_2O.
2. Add 2 ml Solution A. Vortex, let stand for 10 minutes.
3. Add 0.2 ml Solution B. Quickly vortex. Let stand for 30-45 min.

Read the A_{750} and determine the concentration compared to a standard curve.

APPENDIX D

Colorimetric determination of ferrous iron using ortho-phenanthroline

(Krishna Murti et al 1966, Greenberg et al. 1985)

In low pH samples (pH3.5) red colored complex forms rapidly between 3 o-phenanthroline + 1 Fe^{2+}. This complex is very stable and can be determined spectrophotometrically.

Solutions required:

1. Stock ferrous iron solution (200mg/l final concentration)
   -Using ferrous ammonium sulfate (Greenberg et al. 1985)
   a. Slowly add 20ml conc. H_2SO_4 to 50 ml dH_2O
   b. Dissolve 1.404 g $Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$
   c. Dilute to 1 l with water.

2. o-Phenanthroline Reagent (0.2%)
   a. Dissolve 0.50 g 1,10 -phenanthroline monohydrate (Fisher Scientific) in a small amount of dH_2O. Add 5 drops conc. HCl to aid dissolution.
   b. Dilute to 250 ml with water.

3. Sodium acetate-acetic acid buffer, pH 3.5
   a. Dissolve 27.2 g sodium acetate in 500 mL H_2O
   b. Add 123 ml glacial acetic acid.
   c. Dilute to 1 l with water.

4. Buffered o-phenanthroline reagent
   a. Mix separately in a volumetric flask:
      15 ml sodium acetate- acetic acid buffer (pH 3.5)
      5 ml o-phenanthroline (0.2%)
   b. Bring to 100 ml with dH_2O.
   c. Pipet accurate volumes (i.e. 5 or 10 ml) into clean test tubes for dilution of samples for iron analysis.

Apppendix D (continued)

Preparation of standard curve and iron analysis:

Using 1 cm. light path, the range of concentrations of ferrous iron detected is from 0 to 5 ppm or 0 to 500 µg/100 ml.

1. Dilute STOCK IRON (200 mg/l). Add 10 ml/ 100 ml dH_2O to a final concentration of 20 mg/l for Standard iron solution. Make dilutions of this standard iron for a standard curve as follows:

Concentration of ferrous ions		Volume of Standard Iron
(ppm)	(µg/100 ml)	(ml/100 ml vol. flask)
0	0	0
0.4	40	2
1	100	5
2	200	10 (or 5ml/50 ml)
3	300	15
4	400	20 (or 10ml/50ml)
5	500	25

2. Add 15 ml sodium acetate/acetic acid buffer to each flask.
3. Add 5ml o-phenanthroline (0.2%).
4. Dilute to volume.
5. Read A_{510} and make standard curve of A_{510} vs Fe^{2+} concentration.

Samples for Fe^{2+} analysis are diluted into accurate volumes of buffered o-phenanthroline, as for the standard curve. These are read at A_{510} vs. an appropriate blank (i.e. same dilution of an uninnoculated control). The concentration of ferrous ions can be calculated by referring to the standard curve.

APPENDIX E

Table E1. Summary of characteristics of the iron-reducing bacteria and other isolates collected from oil field fluids from May1984- Nov.1986 and the ATCC strains used for comparative purposes

Isolate	Identification	Ferric iron reduction	Sulfide generation from		Nitrate reduction to nitrite	O/F test Acid from glucose	Extracellular hydrolases		
			sulfite	thiosulfate			Gelatin	Starch	DNase
Obuekwe									
Ps 200 (Pem)	*A. putrefaciens*	+	+	+	+	-	+	-	+
213 (Pem)	*A. putrefaciens*	+	-	+	+	+	+	-	+
216 (Pem)	*A. putrefaciens*	+	+	+	+	-	+	-	+
230 (Pem)	*A. putrefaciens*	+	+	+	+	-	+	-	+
Semple									
T2Fed 2	*A. putrefaciens*	+	+	+	+	-	+	-	+
T2Fed 3	*A. putrefaciens*	+	+	+	+	-	+	-	+
T2Fed 4	*A. putrefaciens*	+	+	+	+	-	± *	-	+
T2Pem 12	*A. putrefaciens*	+	+	+	+	-	+	-	+
T3Fed 3	*A. putrefaciens*	+	+	+	+	-	+	-	+
T3Fed 4	*A. putrefaciens*	+	+	+	+	-	+	-	+
T3Fed 8	*A. putrefaciens*	+	+	+	+	-	±	nd **	nd
AN22 (Fed)	*A. putrefaciens*	+	+	+	+	-	+	-	+
AN24 (Red)	*A. putrefaciens*	+	+	+	+	-	+	-	+
AN29 (Fed)	*A. putrefaciens*	+	+	+	+	-	+	-	+
Trip 4									
CL30	*A. putrefaciens*	+	+	+	+	±	+	nd	+
CL31	*A. putrefaciens*	+	+	+	+	±	+	-	+
CL32	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL33	*A. putrefaciens*	+	+	+	+	-	+	-	+
CL36	*A. putrefaciens*	+	+	+	+	-	+	-	+
CL41	*A. putrefaciens*	+	+	+	+	-	+	-	+

Table E1 (continued)

Isolate	Identification	Ferric iron reduction	Sulfide generation from		Nitrate reduction to nitrite	OF test Acid from glucose	Extracellular hydrolases		
			sulfite	thiosulfate			Gelatin	Starch	DNase
Trip 5									
CL47	*A. putrefaciens*	+	+	+	+	-	+	-	+
CL48	*A. putrefaciens*	+	+/- ***	+	+	-	-	-	+
CL49	*A. putrefaciens*	+	+	+	+	-	+	-	+
Trip 6									
Pem 52	*A. putrefaciens*	+	+	+	+	±	+	nd	nd
Pem 53	*A. putrefaciens*	+	+	+	+	-	+	nd	nd
CL56	*A. putrefaciens*	+	+	+	+	-	-	nd	nd
CL57	*A. putrefaciens*	+	+	+	+	-	+	nd	nd
CL58	*A. putrefaciens*	+	+	+	+	-	-	nd	nd
CL59	*A. putrefaciens*	+	+	+	+	-	-	nd	nd
CL60	*A. putrefaciens*	+	+	+	+	-	-	nd	nd
Red T5-3JAN	*A. putrefaciens*	+	+	+	+	-	+	nd	nd
ESSO 1-1	*A. putrefaciens*	+	-	+	+	-	+	-	+
ESSO 1-3	*A. putrefaciens*	+	-	+	+	-	+	-	+
ESSO 4-1	*A. putrefaciens*	+	-	+	+	+	+	-	+
ESSO 4-2	*A. putrefaciens*	+	-	+	+	+	+	-	+
ESSO 1-7	*A. putrefaciens*	+	+/-	+	+	-	+	-	+
ESSO 1-8	*A. putrefaciens*	+	+/-	+	+	-	+	-	+
ESSO 1-2S	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
ESSO 1-2R	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Trip 7									
Pem 61	*A. putrefaciens*	+	-	+	+	-	nd	nd	nd
Pem 62	*A. putrefaciens*	+	-	+	+	-	nd	nd	nd
Fed 63	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 64	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd

Table E1 (continued)

Isolate	Identification	Ferric iron reduction	Sulfide generation from		Nitrate reduction to nitrite	OF test Acid from glucose	Extracellular hydrolases		
			sulfite	thiosulfate			Gelatin	Starch	DNase
Trip 7 continued									
CL 65	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL 66	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL 67	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL 70	*A. putrefaciens*	+	-	+	+	+	+	-	+
CL 71	*A. putrefaciens*	+	+	+	+	-	+	-	+
CL 72	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL 73	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
CL 74	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 75	*A. putrefaciens*	+	+	+	+	-	+	-	+
Fed 76	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 77	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 78	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 79	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 80	*A. putrefaciens*	+	+	+	+	-	+	-	+
Fed 81	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
Fed 82	*A. putrefaciens*	+	+	+	+	-	nd	nd	nd
+ 22 more lost	*A. putrefaciens*	+	+	+	nd	-	nd	nd	nd
ATCC 8071	*A. putrefaciens*	+	+	+	+	-	+	-	+
ATCC 8072	*A. putrefaciens*	+	-	+	+	+	+	-	+
ATCC 8073	*A. putrefaciens*	+	-	+	+	+	+	-	+
ATCC 19857	*P. rubescens*	+	+	+	+	-	+	-	+
ATCC 12099	*P. rubescens*	+	+	+	+	-	+	-	+

Table E1 (continued)

Isolate	Identification	Ferric iron reduction	Sulfide generation from		Nitrate reduction to nitrite	O/F test Acid from glucose	Extracellular hydrolases		
			sulfite	thiosulfate			Gelatin	Starch	DNase
Fermentative isolates									
AN13	Coryneform	+	-	-	-	+/Ferment	-	-	-
AN27	*Enterobacter*	+	-	-	+/gas	+/Ferment	-	-	-
AN28	*Enterobacter*	+	-	-	+/gas	+/Ferment	nd	-	-
Non-iron reducers									
ESSO 2-2	*Pseudomonas*	-	-	-	+	+	nd	+	-
ESSO 4-3	*Pseudomonas*	-	-	-	+	+	nd	+	-
ESSO 5-1	*Pseudomonas*	-	-	-	-	+	nd	-	-
ESSO 7-4	*Pseudomonas*	-	-	-	+	+	nd	nd	nd
ESSO 4-4	*Pseudomonas*	-	-	-	+	+	nd	nd	nd
ESSO 1-4	*Pseudomonas*	-	-	-	+	+	nd	+	-
ATCC 10145	*P. aeruginosa*	-	-	-	+/gas	+	+	-	-
ATCC 13315	*Proteus vulgaris*	-	-	+	+	+/Ferment	nd	-	+
Salt requring Alteromonas sp (ATCC #)									
Pseudomonas atlantica	*19262*	-	-	-	nd	nd	nd	nd	+
Pseudomonas piscicida	*15251*	-	-	-	nd	nd	nd	nd	+
Alteromonas macleodii	*27126*	-	-	-	nd	nd	nd	nd	+
Alteromonas haloplanctis	*14393*	-	-	-	nd	nd	nd	nd	+
Alteromonas rubra	*29570*	-	-	-	nd	nd	nd	nd	+
Alteromonas aurantia	*33046*	-	-	-	nd	nd	nd	nd	no growth

* ± = weak reaction

** nd= not determined

*** +/- = variable reaction

Table E2. Summary of characteristics of the iron-reducing bacteria and other isolates collected from oil field fluids and ATCC strains used for comparative purposes

Isolate	Ornithine decarboxylase	TMAO Reduction	Simmons Citrate	Urease	Growth at 4°C	Growth with NaCl at 25°C 4.5%	Growth with NaCl at 25°C 6.0%	Growth with NaCl at 25°C 7.5%	mol% G+C	Oil Degradation
Obuekwe										
Ps 200 (Pem)	+	+	-	-	+	+	-	+	42.4	Aroms *
213 (Pem)	+	+	-	-	+	+	-	+	44.2	nd**
216 (Pem)	+	+	-	-	+	+	-	+	45.4	nd
230 (Pem)	+	+	-	-	+	+	-	+	nd	nd
Semple										
T2Fed 2	+	+	+	+	-	+	+	+	nd	Aroms
T2Fed 3	+	+	+	+	-	+	+	+	49.4	Aroms
T2Fed 4	+	+	+	-	-	+	+	+	nd	nd
T2Pem 12	+	+	-	+	+	+	-	-	42.8	Aroms
T3Fed 3	+	+	+	+	-	+	+	+	nd	nd
T3Fed 4	+	+	+	+	-	+	+	+	nd	nd
T3Fed 8	+	+	nd	nd	nd	nd	nd	nd	nd	nd
AN22 (Fed)	+	+	-	-	-	+	+	+	55.2	Aroms
AN24 (Red)	+	+	+	+	-	+	+	+	51.4	nd
AN29 (Fed)	+	+	-	-	-	+	+	+	nd	nd
Trip 4										
CL30	+	+	-	nd	-	+	+	± ***	nd	nd
CL31	+	+	-	-	-	+	+	+	54.2	Aroms
CL32	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CL33	+	+	+	-	-	+	+	+	nd	nd
CL36	+	+	-	-	-	+	+	+	nd	nd
CL41	+	+	+	-	-	+	+	+	51.4	Aroms

Table E2 (continued)

Isolate	Ornithine decarboxylase	TMAO Reduction	Simmons Citrate	Urease	Growth at 4°C	Growth with NaCl at 25°C 4.5%	6.0%	7.5%	mol% G+C	Oil Degradation
Trip 5										
CL47	+	+	-	-	-	+	+	±	nd	nd
CL48	+	+	-	-	-	+	+	±	nd	nd
CL49	+	+	-	-	-	+	+	±	nd	nd
Trip 6										
Pem 52	nd	nd	-	nd	-	+	+	±	nd	nd
Pem 53	nd	nd	-	nd	-	+	+	±	nd	nd
CL56	nd	nd	-	nd	-	+	+	+	nd	nd
CL57	nd	nd	-	nd	-	+	+	+	nd	nd
CL58	nd	nd	-	nd	-	+	+	±	nd	nd
CL59	nd	nd	-	nd	-	+	+	±	nd	nd
CL60	nd	nd	-	nd	-	+	+	+	nd	nd
Red T5-3JAN	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ESSO 1-1	+	+	+	-	+	+	-	-	44.8	Aroms
ESSO 1-3	+	+	+	-	-	+	+	-	50.8	Aroms
ESSO 4-1	+	+	+	-	+	+	-	-	43.8	Aroms
ESSO 4-2	+	+	+	-	+	+	+	-	nd	Aroms
ESSO 1-7	+	+	-	+	-	+	+	+	nd	Aroms
ESSO 1-8	+	+	-	+	-	+	+	+	nd	nd
ESSO 1-2S	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ESSO 1-2R	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Trip 7										
Pem 61	nd	nd	+	nd	nd	nd	nd	nd	nd	nd
Pem 62	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fed 63	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fed 64	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table E2 (continued)

Isolate	Ornithine decarboxylase	TMAO Reduction	Simmons Citrate	Urease	Growth at 4°C	Growth with NaCl at 25°C 4.5%	Growth with NaCl at 25°C 6.0%	Growth with NaCl at 25°C 7.5%	mol% G+C	Oil Degradation
Trip 7 continued										
CL 65	nd	nd	+	nd	-	+	+	+	nd	nd
CL 66	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CL 67	nd	nd	-	nd	-	+	+	±	nd	nd
CL 70	+	+	-	-	+	+	-	-	48.6	Aroms
CL 71	+	+	+	+	-	+	+	+	nd	nd
CL 72	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
CL 73	nd	nd	+	nd	-	+	+	+	nd	nd
CL 74	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fed 75	+	+	+	-	-	+	+	+	55.6	Aroms
Fed 76	nd	nd	-	nd	-	+	+	±	nd	nd
Fed 77	nd	nd	-	nd	-	+	+	±	nd	nd
Fed 78	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fed 79	nd	nd	nd	nd	-	+	+	±	nd	nd
Fed 80	+	+	-	+	-	+	+	+	51.8	Aroms
Fed 81	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fed 82	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
+ 22 more lost	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ATCC 8071	+	+	-	-	+	+	-	-	43.8	nd
ATCC 8072	+	+	+	-	+	+	-	-	47.2	nd
ATCC 8073	+	+	-	-	-	+	-	-	46.1	nd
ATCC 19857	+	+	-	-	+	+	-	-	43.6	nd
ATCC 12099	+	+	-	-	+	+	-	-	44.2	nd

Table E2 (continued)

Isolate	Ornithine decarboxylase	TMAO Reduction	Simmons Citrate	Urease	Growth at 4°C	Growth with NaCl at 25°C 4.5%	6.0%	7.5%	mol% G+C	Oil Degradation
Fermentative isolates										
AN13	-	nd	nd	nd	nd	nd	nd	nd	nd	nd
AN27	-	nd	nd	nd	nd	nd	nd	nd	nd	nd
AN28	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Non-iron reducers										
ESSO 2-2	-	-	nd	nd	-	+	+	+	nd	nd
ESSO 4-3	-	-	nd	nd	-	+	+	-	nd	nd
ESSO 5-1	-	-	+	nd	+	±	-	-	nd	nd
ESSO 7-4	-	-	+	nd	-	+	+	-	nd	nd
ESSO 4-4	-	-	+	nd	-	+	+	-	nd	nd
ESSO 1-4	-	-	+	nd	-	+	+	-	nd	nd
P. aeruginosa	-	-	+	+	-	+	+	-	67	nd
P. vulgaris	-	+	-	+	nd	nd	nd	nd	nd	nd
Salt requring Alteromonas										
P. atlantica	-	-	nd	nd	+	+	+	+	43.5	nd
P. piscicida	-	-	nd	nd	-	±	±	-	44,5	nd
A. macleodii	-	-	nd	nd	-	+	+	+	45.6	nd
A. haloplanctis	-	-	nd	nd	+	+	+	+	43.2	nd
A. rubra	-	-	nd	nd	-	nd	nd	nd	46-48	nd
A. aurantia	-	no growth	nd	nd	-	nd	nd	nd	38-43	nd

* Aroms = selective degradation of biphenyl and methylbiphenyl

** nd= not determined

*** ± = slight growth

APPENDIX F

Table F1. Effect of storage at 4°C in 0.1 M phosphate buffer on iron reduction by resting cells of strains of *A. putrefaciens*

Isolate	Storage time (days)	Iron reduction rate mg Fe^{2+}/l /h /ml cells		Viable cell number (/ml)	Optical density (600 nm)
		23°C	30°C		
Ps 200	0	64.4	nd [a.]	8.80×10^8	1.288
	3	52.0	nd	5.17×10^8	1.262
	4	45.8	51.3	1.56×10^8	1.285
	6	36.7	44.8	1.53×10^8	1.290
	11	38.9	44.0	3.78×10^8	1.277
Pem 12	0	58.9	nd	1.09×10^9	1.007
	3	45.0	nd	1.10×10^8	0.961
	4	43.3	46.3	2.77×10^8	0.939
	6	33.6	38.0	1.53×10^8	0.899
	11	26.7	32.4	3.20×10^8	0.813
ESSO 4-1	0	45.1	nd	3.60×10^7	1.118
	3	41.0	nd	NG [b.]	0.992
	4	35.6	31.1	2.26×10^7	0.963
	6	34.2	25.9	2.10×10^7	0.913
	11	17.7	20.1	3.30×10^6	0.781

a. nd= not determined
b. NG= no growth

APPENDIX F (continued)

Table F2. Summary of iron reduction rates of resting cells of strains of *A. putrefaciens* isolated from oil field fluids

		Resting cell suspension			Iron Reduction Rates @30°C			
Isolate	OD 600 at harvest	Storage time (days)	Viable Cell Number (/ml)	Protein content (µg/ml)	Vol. cells (ml)	mg Fe^{2+}/l /hr /mg protein	Avg. [a]	
Ps 200 (early stationary)	1.57	0	9.7×10^8	624 ± 36	1	97.4	156	156
		2	2.0×10^8	613 ± 8	0.5	35.7	117	112 (28%)[b]
					1	67.2	110	
					2	134.3	110	
					3	166.4	-[c]	
		3	1.9×10^8	649 ± 8	1	43.2	67	67 (57%)
Ps 200 (6 h exponential)	0.88	0	1.7×10^8	295 ± 13	0.5	20	-	
					1	45	153	158
					2	97	164	
					3	159	-	
		2	1.2×10^8	318 ± 7	1	42.8	135	147 (7%)
					1.5	75.7	159	
					2	101.8	160	
					3	148.8	-	
Ps 200 (15 h stationary)	1.6	0	3.9×10^9	432 ± 30	0.5	30.8	142	144
					1	63.6	147	
					2	166	-	
					3	280	-	
		2	3.0×10^9	426 ± 20	0.5	21.4	-	
					1	55.2	130	128 (11%)
					2	108.2	126	
					3	176	-	
Pem 12 (early stationary)	1.24	0	2.1×10^9	547 ± 16	1	128	234	234
		2	7.0×10^8	526 ± 8	0.5	38.3	146	145 (38%)
					1	80	152	
					2	144.9	138	
					3	204	-	
		3	8.8×10^8	534 ± 8	1	48.8	91	91 (61%)
Pem 12 (exponential)	0.87	0	nd[d]	446 ± 15	0.5	59.7	268	282
					1	132.3	297	
					1.5	239.2	-	

Table F2 (continued)

Isolate	OD 600 at harvest	Resting cell suspension: Storage time (days)	Viable Cell Number (/ml)	Protein content (µg/ml)	Iron Reduction Rates @30°C: Vol. cells (ml)	mg Fe^{2+}/l /hr /mg protein		Avg.	
T2Fed 3 (15 h early stationary)	0.93	0	no growth	540 ± 20	1	51.5	95	95	
		2	4.0×10^7	542 ± 20	1	2.7	-		
					2	28.6	26	29	(69%)
					3	50	31		
					4	67.1	31		
T2Fed 3 (14 h exponential)	0.92	0	6.0×10^7	306 ± 6	0.5	10.6	-		
					1	22.3	73	94	
					2	61	99		
					3	103.3	112		
		2	2.3×10^7	353 ± 5	1	0	-		
					2	14.4	-		
					3	29	27	29	(69%)
					4	43.5	31		
T2Fed 3 (17 h stationary)	1.2	0	1.8×10^8	469 ± 30	0.5	0	-		
					1	27.2	58	58	
					2	53.7	57		
					3	82	58		
		2	3.4×10^7	464 ± 20	1	0	-		
					2	19.2	21	25	(57%)
					3	34.9	25		
					4	53.6	29		
GROUP 1									
216	0.59	0	2.09×10^9	486 ± 12	1	44.8	92	94	
					1.5	69.1	95		
					2	92.5	95		
		2	nd	512 ± 18	1	23.8	46	51	(46%)
					2	57.5	56		
230	0.88	0	7.0×10^9	674 ± 16	1	107.8	160	160	
					1.5	160.7	-		
					2	215.8	-		
		2	nd	736 ± 18	1	86.9	118	118	(26%)
					2	186.8	-		
A. putrefaciens ATCC 8071	1.05	0	nd	443 ± 12	0.5	27	122	132	
					1	63.3	143		
					1.5	108.5	163		

Table F2 (continued)

Isolate	OD 600 at harvest	Resting cell suspension: Storage time (days)	Viable Cell Number (/ml)	Protein content (µg/ml)	Iron Reduction Rates @30°C: Vol. cells (ml)	mg Fe^{2+}/l /hr /mg protein		Avg.	
GROUP 2									
213	1.36	0	2.6×10^7	742 ± 19	1	87.5	118	118	
					1.5	151.9	-		
					2	193.5	-		
		2	4.2×10^8	744 ± 25	1	74.8	101	105	(11%)
					1.5	123	110		
					2	149.6	101		
CL70	1.37	0	nd	661 ± 21	0.5	39.1	-	184	
					1	121.4	184		
					1.5	211.6	-		
					2	272.4	-		
ESSO 4-1	1.30	0	4.6×10^8	479 ± 13	1	72.1	151	167	
					1.5	131.3	183		
					2	174.2	182		
		2	2.9×10^8	462 ± 6	1	34.7	75	84	(50%)
					1.5	64.2	93		
					2	92.1	100		
ESSO 1-1	1.45	0	7.0×10^9	668 ± 17	1	142	213	213	
					1.5	185.9	-		
					2	255.8	-		
		2	2.8×10^9	685 ± 17	1	47	69	65	(69%)
					1.5	64	62		
					2	102	74		
ESSO 4-2	0.80	0	5.3×10^8	498 ± 12	1	74.5	150	153	
					1.5	116.4	156		
					2	158.4	159		
		2	nd	510 ± 5	1	51	100	100	(35%)
					2	156	-		
GROUP 3									
ESSO 1-3	1.60	0	nd	527 ± 28	1	125.2	238	238	
					1.5	186.8	-		
					2	271.8	-		
		2	nd	570 ± 11	1	52.6	92	103	(57%)
					1.5	97.6	114		
					2	126.9	111		

Table F2 (continued)

Isolate	OD 600 at harvest	Resting cell suspension: Storage time (days)	Viable Cell Number (/ml)	Protein content (µg/ml)	Iron Reduction Rates @30°C: Vol. cells (ml)	mg Fe^{2+}/l /hr /mg protein		Avg.	
GROUP 4									
CL41	1.28	0	3.3×10^7	474 ± 16	1	44.8	92	92	
		2	2.0×10^7	461 ± 10	1	0	-		
					2	1.8	-		
					3	20.3	15	14	(85%)
					4	23.9	13		
CL31	1.29	0	nd	478 ± 16	0.5	45.5	190	177	
					1	78.2	164		
					1.5	128.7	179		
					2	174.4	-		
AN22	1.39	0	nd	563 ± 42	0.5	33.9	120	145	
					1	86.6	154		
					1.5	136.7	162		
					2	159.2	-		
CL71	1.17	0	nd	512 ± 16	0.5	16	-		
					1	45.6	89	106	
					1.5	95.1	124		
					2	124.1	121		
AN24	1.16	0	nd	400 ± 26	0.5	4.1	-		
					1	13.1	33	42	
					1.5	26.6	44		
					2	38.7	48		
Fed 75	1.45	0	nd	627 ± 35	0.5	38.3	122	140	
					1	99.5	159		
					1.5	143.8	-		
					2	182.8	-		
Fed 80	0.74	0	nd	389 ± 6	0.5	10.1	-		
					1	25.4	65	75	
					1.5	47.4	81		
					2	61.6	79		

a. Iron reduction rates per unit protein were determined for rates between 20 and 130 mg Fe(II)/l /h. Activity expressed as µmol Fe(II)/h/mg protein can be calculated by dividing by a factor of 5.58

b. ( ) = % decrease in rate from Day 0 after storage

c. - = not calculated

d. nd = not determined

APPENDIX G

Calculations used for sulfur isotope fractionation data

1. Reservoir $\delta^{34}S$ value (McCready and Krouse 1979)

-calculate the value of the reservoir sulfur at time t_i using the $\delta^{34}S$ values of the evolved sulfide fractions

$$\delta i_{res} = \frac{-\sum_{1}^{i} X_i \ \delta_i}{100 - \sum_{1}^{i} X_i}$$

Where $\delta i_{res} = \delta^{34}S$ value of the reservoir at time t_i

δ_i = the $\delta^{34}S$ value of the i th fraction of H_2S evolved

X_i = the percentage of sulfide in the i th fraction of H_2S, collected from t_{i-1} to t_i

100 = the total percentage of S in $SO_3^=$ at time zero

2. $\delta^{34}S$ of the H_2S (accummulated) (Krouse et al. 1968)

-refers to the average isotopic composition of all H_2S collected up to a given point in the reaction

$$\delta_{accumulated} = \frac{\Sigma \delta_{34} \text{ (fraction i) x mass i}}{\text{Total mass of product collected}} = \frac{\Sigma \delta_i \ X_i}{X \text{ Total}}$$

APPENDIX G (continued)

Table G-1. Kinetics and sulfur isotope fractionation during anaerobic reduction of sulfite by growing cells of *A. putrefaciens*: isolate Ps 200.

Sample Number	Growth Time	% Reaction	$\delta^{34}S$ (H_2S Fraction)	$\delta^{34}S$ (H_2S Accumulated)	$\delta^{34}S$ (Reservoir Calculated)
1	8	3.1	-12.8	-12.8	+0.41
2	9.5	5.4	-14.2	-13.4	+0.76
3	11	11.1	-13.4	-13.4	+1.7
4	12.5	19.7	-13.4	-13.4	+3.3
5	14	32.8	-13.4	-13.4	+6.5
6	15	38.6	-5.4	-12.2	+7.7
7	16	47.6	-0.6	-10.0	+9.0
8	17	56.2	+6.7	-7.4	+9.5
9	18.5	63.6	+11.2*	-5.3	+9.2
10	20.5	69.4	+10.0*	-3.9	+9.2
11	21.5	71.8	+9.9*	-3.5	+9.0
12	32.5	81.1	+6.9	-2.3	+10.0
13	40.5	81.7	+5.5	-2.2	+10.1
14	57.5	83.5	+8.0	-2.0	+10.4
15	63	83.8	+12.5*	-1.95	+10.3

*Inverse isotope effect is evident where H_2S evolved is enriched in ^{34}S as compared to the total sulfur remaining in the reaction solution (higher $\delta^{34}S$ than the reservoir)

APPENDIX G (continued)

Table G-2. Kinetics and sulfur isotope fractionation during anaerobic reduction of sulfite by growing cells of *A. putrefaciens*: Isolate T2Fed 3

Sample Number	Growth Time	% Reaction	$\delta^{34}S$ (H_2S Fraction)	$\delta^{34}S$ (H_2S accumulated)	$\delta^{34}S$ (Reservoir Calculated)
1	11	0.1	-8.7	-8.7	+0.01
2	13	2.1	-13.3	-13.1	+0.28
3	15	3.7	-12.5	-12.8	+0.49
4	17	6.6	-12.2	-12.6	+0.88
5	18.5	10.0	-11.4	-12.2	+1.3
6	20	14.8	-10.9	-12.2	+2.0
7	12.5	21.5	-9.3	-11.0	+3.0
8	23	29.6	-5.3	-9.4	+4.0
9	24.5	37	-3.8	-8.6	+4.9
10	33.5	70.9	+3.8	-2.6	+6.1
11	38	75.0	+20.2*	-1.4	+3.8
12	42	77.5	+20.0*	-0.7	+2.0

*Inverse isotope effect is evident where H_2S evolved is enriched in ^{34}S as compared to the total sulfur remaining in the reaction solution (higher $\delta^{34}S$ than the reservoir)

APPENDIX G (continued)

Table G-3. Kinetics and sulfur isotope fractionation during anaerobic reduction of $SO_3^=$ by growing cells of *A. putrefaciens*: Isolate Pem 12

Sample Number	Growth Time	% Reaction	$\delta^{34}S$ (H_2S Fraction)	$\delta^{34}S$ (H_2S accumulated)	$\delta^{34}S$ (Reservoir Calculated)
1	11	0.3	-2.7	-2.7	0
2	13	0.4	-4.3	-3.1	0
3	14	1.2	-5.1	-4.4	+0.05
4	15	1.6	-5.8	-4.8	+0.07
5	16	2.3	-6.5	-5.3	+0.12
6	17	3.3	-7.4	-5.9	+0.2
7	18	4.6	-9.2	-6.8	+0.33
8	19	6.4	-10.9	-8.0	+0.55
9	20	7.7	-12.1	-8.7	+0.73
10	21	10.6	-13.1	-9.9	+1.2
11	22	14.8	-12.5	-10.6	+1.85
12	23	20.9	-13.0	-11.3	+2.99
13	24.5	27.2	-13.6	-11.8	+4.43
14	32.5	60.2	-7.8	-9.6	+14.6
15	34	63.4	-1.1	-9.2	+15.9
16	35	67.4	+3.1	-8.5	+17.5
17	39	72.7	+11	-7.0	+18.8
18	41.5	76.4	+21.3*	-5.7	+18.4
19	46	80.8	+34.2*	-3.5	+14.8
20	57	83.5	+32.7*	-2.3	+11.8
21	64	84.1	+27.9*	-2.1	+11.2

*Inverse isotope effect is evident where H_2S evolved is enriched in ^{34}S as compared to the total sulfur remaining in the reaction solution (higher $\delta^{34}S$ than the reservoir)

APPENDIX G (continued)

Table G-4. Kinetics and sulfur isotope fractionation during anaerobic reduction of sulfite by growing cells of *A. putrefaciens*: Isolate CL41

Sample Number	Growth Time	% Reaction	$\delta^{34}S$ (H_2S Fraction)	$\delta^{34}S$ (H_2S accumulated)	$\delta^{34}S$ (Reservoir Calculated)
1	11	2.3	-7.7	-7.7	0.18
2	13	4.3	-10.9	-9.2	0.41
3	15	7.6	-14.0	-11.3	0.93
4	16.5	11.8	-12.9	-11.8	1.59
5	18	16.9	-10.9	-11.6	2.35
6	19	22.1	-10.4	-11.3	3.2
7	20	29.9	-12	-11.5	4.9
8	21	40.0	-9.2	-10.9	7.3
9	22.5	52.7	-4.3	-9.3	10.4
10	25	63.5	0.3	-7.7	13.3
11	34	78.6	8.1	-4.6	17.1
12	38	80.6	11.3	-4.2	17.7
13	41	82.3	11.5	-3.9	18.2
14	58	85.2	11.7	-3.4	19

APPENDIX H

Table H1. Differences in the corrosion rates and the amount of corrosion product on mild steel corrosion coupons in corrosion experiments with mixed and pure cultures of isolates *A. putrefaciens* Ps 200 and *D. vulgaris* AL1

Corrosion Test Inoculum and medium	Corrosion rate (mdd)	Estimated weight of attached material [a.] (mg)	Description of corrosion products and coating	Figure
Ps 200				
Butlin's	2.1	9.8	- no coating and little attachment	15,16
-YE	4.6	58.1	- white coating turns blue in air, crystalline (iron phosphate)	15,17
$+S_2O_3^=$	2.0	13.0	- thin black, protective FeS layer	15,16
AL1				
Butlin's 6 wk	11.6	92.6	-heavy black slime, mats of long	18,19
12wk	3.3	62.1	rods interspersed with FeS, more at 6 weeks	
-YE	18.6	58.4	- heavy black slime, only developed from 6 wks, rinsed off easily	18,20
$+S_2O_3^=$	15.0	215.4	- very heavy black slime	18,19
Mixed culture				
Butlin's	1.1	14.0	- thin black adherent FeS layer thin layer of attached organisms	21,22
-YE	22.7	246.5	- very heavy black slime	21,22
$+S_2O_3^=$	3.6	24.0	- nonadherent mucoid gray coating, and protective FeS film	23
Control				
Butlin's	3.8	100.6	-yellow, amorphous oxide	14
- YE	4.3	105.1	-yellow, amorphous oxide	
$+S_2O_3^=$	9.1	183.8	- yellow coat with black patches and black undercoat	

a. Approximate amount of corrosion product on fixed and dried coupons calculated from the difference between the weight of coupon with attached corrosion product and the average weight loss after acid cleaning. Calculations shown in Table H2.

APPENDIX H (continued)

Table H2. Weight gain and estimation of the amount of corrosion product attached to corrosion coupons after fixing and drying

Corrosion Test Medium and inoculum	Time (weeks)	Corrosion coupons: Weight gain of fixed and dried coupon [a] (mg)	Corrosion coupons: Avg. weight loss after acid cleaning [b] (mg)	Approximate amount of attached material [c] (mg)
Butlin's				
Control	6	43.1	32.2	75.3
	12	62.7	37.9	100.6
Ps 200	6	-9.7	12.9	3.2
	12	-11.1	20.9	9.8
AL1	6	35.3	57.3	92.6
	12	28.0	34.1	62.1
Mixed	6	1.0	8.5	9.5
	12	3.1	10.9	14.0
Butlin's -YE				
Control	12	62.4	42.7	105.1
Ps 200	12	12.4	45.7	58.1
AL1	12	-126.0	184.4	58.4
Mixed	12	22.0	224.5	246.5
Butlin's $+S_2O_3^=$				
Control	12	93.8	90.0	183.8
Ps 200	12	-6.7	19.7	13.0
AL1	12	66.5	148.9	215.4
Mixed	12	-11.4	35.4	24.0

a. Negative values indicate a weight loss.
b. Average weight loss of 3 coupons, as per Table 18.
c. Total corrosion product = Avg. weight loss after acid cleaning + weight gain after drying. Difference between the weight with attached corrosion product and the weight after acid cleaning is the approximate amount of corrosion product .